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(12) **United States Patent**  
**Robinson et al.**(10) **Patent No.:** **US 7,795,017 B2**(45) **Date of Patent:** **Sep. 14, 2010**(54) **DNA EXPRESSION VECTORS AND METHODS OF USE**(75) Inventors: **Harriet L. Robinson**, Atlanta, GA (US);  
**Rama R. Amara**, Atlanta, GA (US); **Ted M. Ross**, Aspinall, PA (US); **Rick A. Bright**, Atlanta, GA (US)(73) Assignee: **Emory University**, Atlanta, GA (US)

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**C12N 15/63** (2006.01)(52) **U.S. Cl.** ..... **435/320.1**; 536/23.1(58) **Field of Classification Search** ..... None  
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**

5,169,763 A 12/1992 Kieny et al.  
 5,185,146 A 2/1993 Altenburger  
 5,256,767 A 10/1993 Salk et al.  
 5,445,953 A 8/1995 Dorner et al.  
 5,494,807 A 2/1996 Paoletti et al.  
 5,589,466 A 12/1996 Felgner et al.  
 5,593,972 A 1/1997 Weiner et al.  
 5,614,404 A 3/1997 Mazzara et al.  
 5,676,950 A 10/1997 Small et al.  
 5,736,368 A 4/1998 Mazzara et al.  
 5,741,492 A 4/1998 Hurwitz et al.  
 5,747,324 A 5/1998 Mazzara et al.  
 5,747,338 A 5/1998 Giese et al.  
 5,756,103 A 5/1998 Paoletti et al.  
 5,766,599 A 6/1998 Paoletti et al.  
 5,795,577 A 8/1998 Kieny et al.  
 5,817,637 A 10/1998 Weiner et al.  
 5,846,946 A 12/1998 Huebner et al. .... 514/44  
 5,849,304 A 12/1998 Moss et al.  
 5,853,725 A 12/1998 Salk et al.  
 5,858,775 A \* 1/1999 Johnson ..... 435/320.1  
 5,863,542 A 1/1999 Paoletti et al.  
 5,879,925 A 3/1999 Rovinski et al.  
 5,911,989 A 6/1999 Katinger et al. .... 424/160.1  
 5,928,930 A 7/1999 Salk et al.  
 5,985,641 A 11/1999 Haynes et al.  
 6,051,410 A 4/2000 Mazzara et al.

6,077,662 A 6/2000 Compans et al. .... 435/5  
 6,080,408 A 6/2000 Rovinski et al.  
 6,086,891 A 7/2000 Hurwitz et al.  
 6,099,847 A 8/2000 Tobin et al.  
 6,103,244 A 8/2000 Dorner et al.  
 6,121,021 A 9/2000 Rovinski et al.  
 6,140,114 A 10/2000 Klatzmann et al.  
 6,156,952 A 12/2000 Bryant et al. .... 800/11  
 6,171,596 B1 1/2001 Earl et al.  
 6,204,250 B1 3/2001 Bot et al.  
 6,210,663 B1 4/2001 Ertl  
 6,214,804 B1 4/2001 Felgner et al.  
 6,265,183 B1 7/2001 Dorner et al.  
 6,291,157 B1 9/2001 Rovinski et al.  
 6,448,083 B1 \* 9/2002 Larocca et al. .... 435/456

(Continued)

**FOREIGN PATENT DOCUMENTS**

EP 0 335 635 10/1989

(Continued)

**OTHER PUBLICATIONS**

Letvin (1998, Science, vol. 280, pp. 1875-1880).\*

(Continued)

*Primary Examiner*—Celine X Qian(74) *Attorney, Agent, or Firm*—Fish & Richardson P.C.(57) **ABSTRACT**

The present invention relates to novel plasmid constructs useful for the delivery of DNA vaccines. The present invention provides novel plasmids having a transcription cassette capable of directing the expression of a vaccine nucleic acid insert encoding immunogens derived from any pathogen, including fungi, bacteria and viruses. The present invention, however, is particularly useful for inducing in a patient an immune response against pathogenic viruses such as HIV, measles or influenza. Immunodeficiency virus vaccine inserts of the present invention express non-infectious HIV virus-like particles (VLP) bearing multiple viral epitopes. VLPs allow presentation of the epitopes to multiple histocompatibility types, thereby reducing the possibility of the targeted virus escaping the immune response. Also described are methods for immunizing a patient by delivery of a novel plasmid of the present invention to the patient for expression of the vaccine insert therein. Optionally, the immunization protocol may include a booster vaccination that may be a live vector vaccine such as a recombinant pox virus or modified vaccinia Arbora vector. The booster live vaccine vector includes a transcription cassette expressing the same vaccine insert as the primary immunizing vector.

**10 Claims, 61 Drawing Sheets**

## U.S. PATENT DOCUMENTS

6,544,527 B1 4/2003 Rovinski et al. .... 424/208.1  
 6,663,871 B1 12/2003 McMichael et al.  
 6,841,381 B1\* 1/2005 Robinson et al. .... 435/320.1

## FOREIGN PATENT DOCUMENTS

EP 0 538 496 8/1991  
 WO WO 89/09620 10/1989  
 WO WO 89/12095 12/1989  
 WO WO 91/07425 5/1991  
 WO WO 97/27311 7/1997  
 WO WO 98/56919 12/1998  
 WO WO 99/63098 12/1999  
 WO WO 00/00216 1/2000  
 WO WO 01/47955 7/2000  
 WO WO 01/02607 1/2001  
 WO WO 01/52886 7/2001  
 WO WO 01/82962 11/2001  
 WO WO 01/92470 12/2001  
 WO WO02/072754 9/2002  
 WO WO03/004657 1/2003

## OTHER PUBLICATIONS

Feinberg et al. (2002, *Nature Medicine*, vol. 8, pp. 207-210).  
 Burton et al. Why do we not have an HIV vaccine and how can we make one? *Nature Medicine*, 1998, vol. 4, pp. 495-498.\*  
 Markmeyer et al (*Gene*, 1990, vol. 93, pp. 129-134).  
 Sipsas et al (*J. Clin. Invest.* 1997, vol. 99, No. 4, pp. 752-762).  
 Huang et al., "Human Immunodeficiency Virus Type 1-Specific Immunity . . ." *J. of Virology* 75:4947-4951, 2001.  
 Kong et al., "Immunogenicity of Multiple Gene and Clade Human Immunodeficiency . . ." *J. of Virology* 77(23):12764-12772, 2003.  
 Megede et al., "Increased Expression and Immunogenicity of Sequence-Modified . . ." *J. of Virology* 74(6):2628-2635, 2000.  
 Persson et al., "Modifications of HIV-1 Retrovirus-Like Particles to Enhance . . ." *Biologicals* 26:255-265, 1998.  
 Smith et al., "Multiprotein HIV Type 1 Clade B DNA/MVA Vaccine: . . ." *AIDS Research and Human Retroviruses* 20(6):654-665, 2004.  
 Accession No. AF430344; (XP-002321209); Smith et al., Oct. 9, 2001.  
 Accession No. AF426288; (XP-002321210); Smith et al., Mar. 12, 2002.  
 Belyakov et al., "Induction of a Mucosal Cytotoxic T-Lymphocyte Response by . . ." *J. of Virology* 72(1):8264-8272, 1998.  
 Davison et al., "Structure of Vaccinia Virus Early Promoters" *J. Mol. Biol.* 210:749-769, 1989.  
 Earl, P.L. et al., "Comparison of vaccine strategies using recombinant *env-gag-pol* MVA with or without an oligomeric *env* protein boost in the SHIV rhesus macaque model" *Virology* (2002) 294:270-281.  
 Girard, M. et al., "New prospects for the development of a vaccine against human immunodeficiency virus type 1. An overview" *C.R. Acad. Sci. Paris, Sciences de la Vie/Life Sciences* (1999) 322:959-966.  
 Gomez, C.E. et al., 2001 "Recombinant proteins produced by vaccinia virus vectors can be incorporated within the virion (IMV form) into different compartments" *Arch Virol.* 146(5):875-892.  
 Haffar et al., "The Carboxy Terminus of Human Immunodeficiency Virus Type I gp 160 . . ." *J. of Virology* 64(6):3100-3103, 1990.  
 Hanke et al., "Development of a DNA-MVA/HIVA vaccine for Kenya" *Vaccine* 20:1995-1998, 2002.  
 Hanke et al., "Effective induction of HIV-specific CTL by multi-epitope using gene gun . . ." *Vaccine* 17:589-596, 1999.  
 Hanke et al., "Immunogenicities of intravenous and intramuscular administrations of modified vaccinia . . ." *J. of General Virology* 79:83-90, 1998.  
 Hanke et al., "Lack of toxicity and persistence in the mouse associated with administration of candidate . . ." *Vaccine* 21:108-114, 2002.  
 Hanke et al., "Pre-clinical development of a multi-CTL epitope-based DNA prime MVA boost vaccine for ADS" *Immunology Letters* 66:177-181, 1999.

Hirsch, V.M. et al., 1995 "Limited virus replication following SIV challenge of macaques immunized with attenuated MVA vaccinia expressing SIVsm *env* and *gag-pol*" *Vaccines* 95:195-200.  
 Karacostas, V. et al., 1989 "Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector" *PNAS USA* 86:8964-8967.  
 Masternak et al., "cis- and trans-Acting Elements Involved in Reactivation of Vaccinia Virus Early Transcription" *J. of Virology* 70(12):8737-8746, 1996.  
 Men, R. et al., "Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge" *Vaccine* (2000) 18:3113-3122.  
 Moss, B. et al., 2000 "Retroviruses of human AIDS and related animal diseases" in: *Colloque des Cent Gardes*, 12<sup>th</sup>, Paris, France, Oct. 25-27, 1999, Meeting Date 1999, 105-107, Eds. M. Girard & B. Dodet, Editions Scientifiques et Medicales Elsevier, Paris, Fr. (Abstract).  
 Wee et al., "A DNA/MVA-based candidate human immunodeficiency virus for Kenya induces . . ." *J. of General Virology* 83:75-80, 2002.  
 Wyatt et al., "Development of a replication-deficient recombinant vaccinia virus vaccine effective against . . ." *Vaccine* 14(15):1451-1458, 1996.  
 Wyatt et al., "Priming and boosting immunity to respiratory syncytial virus by recombinant replication-defective . . ." *Vaccine* 18:392-397, 2000.  
 Gao et al., "Effects of Mutations in the Polymerase Domain on the Polymerase, . . ." *J. Mol. Biol.* 277:559-572, 1998.  
 Ayyavoo et al., "Immunogenicity of a novel DNA vaccine cassette expressing multiple . . ." *AIDS* 14:1-9, 2000.  
 Caselli et al., "DNA immunization with HIV-1 tat mutated in the trans activation domain . . ." *J. of Immunol.* 162:5631-5638, 1999.  
 Englund et al., "Integration is required for productive infection of monocyte-derived macrophages . . ." *J. of Virol.* 69(5):3216-3219, 1995.  
 Le Grice et al., "Active site mutagenesis of the AIDS virus protease and its alleviation . . ." *The EMBO J.* 7(8):2547-2553, 1988.  
 Mizrahi et al., "Mutagenesis of the conserved aspartic acid 443, glutamic acid 478 . . ." *J. of Biol. Chem* 269(30):19245-19249, 1994.  
 Wakefield et al., "In vitro enzymatic activity of human immunodeficiency virus type 1 . . ." *J. of Virol.* 66(11):6806-6812, 1992.  
 Zhang et al., "Nucleocapsid protein effects on the specificity of retrovirus RNA encapsidation" *J. of Virol.* 69(9):5716-5722, 1995.  
 Azevedo et al., "Main features of DNA-based immunization vectors" *Br. J. of Medic. & Biol. Res.* 32:147-153, 1999.  
 Bowie et al., "Deciphering the message in protein sequences: tolerance to amino acid substitutions" *Science* 257:1306-1310, 1990.  
 Davison et al., "New vaccinia virus recombination plasmids incorporating a synthetic . . ." *Nucl. Acids Res.* 18(14):4285-4286, 1990.  
 Earl et al., Immunogenicity and protective efficacy of oligomeric human . . . *J. Virol.* 75(2):645-653, 2001.  
 Greenspan et al., "Defining epitopes: it's not as easy as it seems" *Nature Biotech.* 7:936-937, 1999.  
 Allen et al., "Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen." *J. Immunol.*, 164: 4968-4978, 2000.  
 Amara et al., "Control of a Mucosal Challenge and Prevention of AIDS by a Multiprotein DNA/MVA Vaccine," *Science*, 292: 69-74, 2001.  
 Andre et al., "Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage," *J. Virol.*, 72: 1497-1503, 1998.  
 Antoine et al., "The Complete Genomic Sequence of the Modified Vaccinia Ankara Strain: Comparison with Other Orthopoxviruses," *Virology*, 244: 365-96, 1998.  
 Asakura et al., "Induction of HIV 1 specific mucosal immune responses by DNA vaccination." *Scand. J. Immunol.*, 46: 326-330, 1997.  
 Bachmann and Zinkernagel, "Neutralizing antiviral B cell responses." in *Ann. Rev. Immunol.*, 15: 235-270, 1997.

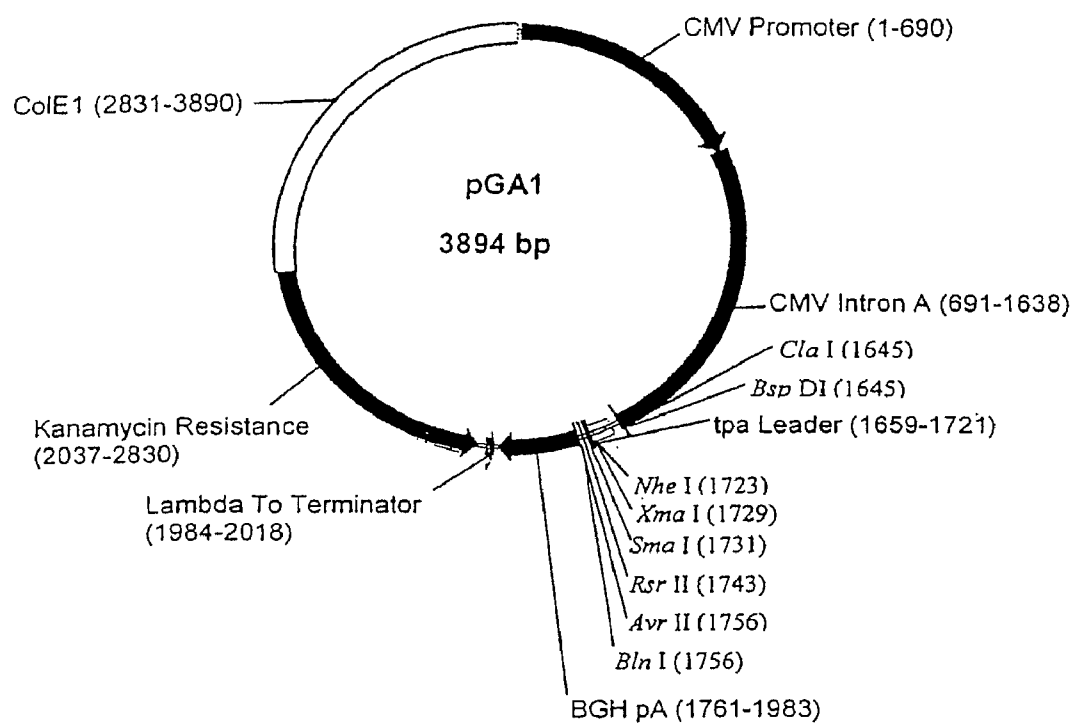
- Barouch et al., "Reduction of Simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination," *J. Virol.*, 75: 5151-5158, 2001.
- Barouch et al., "Control of Viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination," *Science*, 290: 486-492, 2000.
- Barouch et al., "Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/IG plasmid administration in rhesus monkeys", *Proc. Natl. Acad. Sci. U.S.A.*, 97:4192-7, Apr. 11, 2000.
- Barry et al., "Protection against mycoplasma infection using expression-library immunization," *Nature*, 377: 632-635, 1995.
- Berger, "HIV Entry and Tropism: the chemokine receptor connection," *AIDS*, 11(Suppl. A): S3-16, 1997.
- Benson et al., *J. Virol.*, "Recombinant vaccine-induced protection against the highly pathogenic simian immunodeficiency virus SIV(mac251): dependence on route of challenge exposure," 72: 4170-4182, 1998.
- Blanchard et al., "Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine," *J. Gen. Virol.*, 79: 1159-1167, 1998.
- Bohm et al., "DNA vector constructs that prime hepatitis B surface antigen-specific cytotoxic T lymphocyte and antibody responses in mice after intramuscular injection," *J. Immuno. Methods*, 193: 29-40, 1996.
- Bohm et al., "Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses," *Vaccine*, 16: 949-54, 1998.
- Bolivar et al., "Construction and Characterization of New Cloning Vehicles: (II. A Multipurpose Cloning System)," *Gene*, 2: 95-113, 1977.
- Boyer et al., "Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination," *Nature Med.*, 3: 526-532, 1997.
- Boyle et al., "Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization," *Int. Immunol.*, 9: 1897-1906, 1997.
- Boyle et al., "Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction," *Nature*, 392: 408-411, 1998.
- Burton and Montefiori, "The antibody response in HIV-1 infection," *AIDS*, 11(Suppl A):S87-98, 1997.
- Calarota et al., "Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients," *Lancet*, 351: 1320-1325, 1998.
- Cardoso et al., "Immunization with Plasmid DNA Encoding for the Measles Virus Hemagglutinin and Nucleoprotein Leads to Humoral and Cell-Mediated Immunity," *Virology*, 225: 293-299, 1998.
- Carroll and Moss, "Heat Range and Cytopathogenicity of the Highly Attenuated MVA Strain of Vaccinia Virus: Propagation and Generation of Recombinant Viruses in a Nonhuman Mammalian Cell Line", *Virology*, 238:198-211, 1997.
- Chapman et al., "Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells," *Nucl. Acids Res.*, 19: 3979-3986, 1991.
- Chen et al., "Protective Immunity Induced by Oral Immunization with a Rotavirus DNA Vaccine Encapsulated in Microparticles," *J. Virol.*, 72: 5757-5761, 1998.
- Chun et al., "Early establishment of a pool of latently infected, resting CD4+ T cells during primary HIV-1 infection," *Proc. Natl. Acad. Sci. USA*, 95: 8869-8873, 1998.
- Collman et al., "An Infection Molecular Clone of an Unusual Microphage-Tropic and Highly Cytopathic Strain of Human Immunodeficiency Virus Type 1," *J. Virol.*, 66: 7517-7521, 1992.
- Condon et al., "DNA-based immunization by in vivo transfection of dendritic cells," *Nat Med.*, 2:1122-1128, 1996.
- Corr et al., "Gene Vaccination with Naked Plasmid DNA: Mechanism of CTL Priming," *J. Exp. Med.*, 184: 1555-1560, 1996.
- Dempsey et al., "C3d of Complement as a Molecular Adjuvant: Bridging Innate and Acquired Immunity," *Science*, 271: 348-350, 1996.
- Durbin et al., "Comparison of the Immunogenicity and efficacy of a replication-defective vaccinia virus expressing antigens of human parainfluenza virus type 3 (HPIV3) with those of a live attenuated HPIV3 vaccine candidate in rhesus monkeys passively immunized with PIV3 antibodies," *J. Infect. Dis.*, 179: 1345-1351, 1999.
- Durbin et al., "The immunogenicity and efficacy of intranasally or parenterally administered replication-deficient vaccinia-parainfluenza virus type 3 recombinants in rhesus monkeys", *Vaccine*, 16: 1324-30, 1998.
- Egan et al., "Simian immunodeficiency virus (SIV) gag DNA-vaccinated rhesus monkeys develop secondary cytotoxic T-lymphocyte responses and control viral replication after pathogenic SIV infection," *J Virol.*, 74:7485-7495, 2000.
- Endo et al., "Short- and Long-term Clinical Outcomes in Rhesus Monkeys Inoculated with a Highly Pathogenic Chimeric Simian/ Human Immunodeficiency Virus", *J. Virol.*, 74:6935-45, 2000.
- Esparza and Bhamarapravati, "Accelerating the development and future availability of JTV-1 vaccines: why, when, where, and how?", *Lancet*, 355: 2061-6, 2000.
- Evans DT et al., "Virus-specific T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef," *Nat. Med.*, 5: 1270-1276, 1999.
- Feltquate et al., "Different T Helper Cell Types and Antibody Isotypes Generated by Saline and Gene Gun DNA Immunization," *J. Immunol.* 158: 2278-2284, 1997.
- Finzi et al., "Latent infection of CD4 T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy", *Nat. Med.* 5: 1270-6, 1996.
- Fomsgaard et al., "Improved Humoral and Cellular Immune Responses Against the gp120 V3 Loop of HIV-1 Following Genetic Immunization with a Chimeric DNA Vaccine Encoding the V3 Inserted into the Hepatitis B Surface Antigen," *Scand. J. Immunol.*, 47:289-295, 1998.
- Fu et al., "Priming of Cytotoxic T Lymphocytes by DNA Vaccines: Requirement for Professional Antigen Presenting Cells and Evidence for Antigen Transfer from Myocytes," *Mol. Med.*, 3: 362-371, 1997.
- Furci et al., "Antigen-driven C-C Chemokine-mediated HIV-1 Suppression by CD4 T Cells from Exposed Uninfected Individuals Expressing the Wild-type CCR-5 Allele," *J. Exp. Med.*, 186:455-60, 1997.
- Fynan et al., "DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations," *Proc. Natl. Acad. Sci. USA*, 90: 11478-11482, 1993.
- Gorelick et al., "Nucleocapsid Protein Zinc-Finger Mutants of Simian Immunodeficiency Virus Strain Mne Produce Virions That are Replication Defective in Vitro and in Vivo", *Virology*, 259-70, 1999.
- Goulder et al., "Anti-HIV cellular immunity: recent advances towards vaccine design", *AIDS*, 13: S121-36, 1999.
- Hakim et al., "A Nine-Amino Acid Peptide from IL-1 $\beta$  Augments Antitumor Immune Responses Induced by Protein and DNA Vaccines," *J. Immunol.*, 157: 5503-5511, 1996.
- Hanke, "Effective Induction of Simian Immunodeficiency Virus-Specific Cytotoxic T Lymphocytes in Macaques by Using a Multiepitope Gene and DNA Prime-Modified Vaccinia Virus Ankara Boost Vaccination Regimen," *J. Virol.*, 73: 7524-7532, 1999.
- Hanke et al., "Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime," *Vaccine*, 16: 439-445, 1998a.
- Hanke et al., "DNA multi-CTL epitope vaccines for HIV and *Plasmodium falciparum*: immunogenicity in mice," *Vaccine*, 16: 426-435, 1998b.
- Hartikka et al., "An Improved Plasmid DNA Expression Vector for Direct Injection into Skeletal Muscle," *Hum. Gen. Therapy*, 7: 1205-1217, 1996.
- Hirsch et al., "Prolonged Clinical Latency and Survival of Macaques Given a Whole Inactivated Simian Immunodeficiency Virus Vaccine", *J. Infect. Dis.*, 170:51-9, 1994.
- Hofmann-Lehmann et al., "Sensitive and robust one-tube real-time reverse Transcriptase-polymerase chain reaction to quantify SIV RNA load: comparison of one- versus two-enzyme systems," *AIDS Res. Hum. Retroviruses*, 16: 1247-1257, 2000.
- Inchauspe et al., "Plasmid DNA Expressing a Secreted or a Nonsecreted Form of Hepatitis C Virus Nucleocapsid: Comparative Studies of Antibody and T-Helper Responses Following Genetic Immunization," *DNA Cell Biol.*, 16: 185-195, 1997.

- Iwasaki et al., "Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines," *J. Immunol.*, 158: 4591-4601, 1997a.
- Iwasaki et al., "The dominant role of bone-marrow derived cells in CTL induction following plasmid DNA immunization at different sites," *J. Immunol.*, 159: 11-14, 1997b.
- Jin et al., "Dramatic Rise in Plasma Viremia after CD8 T Cell Depletion in Simian Immunodeficiency Virus-infected Macaques," *J. Exp. Med.*, 189: 991-8, 1999.
- Jones et al., "Poly (DL-lactide-co-glycolide) encapsulated plasmid: DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration," *Vaccine*, 15: 814-817, 1997.
- Karlsson et al., "Characterization of Molecularly Cloned Simian-Human Immunodeficiency Viruses Causing Rapid CD4 Lymphocyte Depletion in Rhesus Monkeys," *J. Virol.*, 71: 4218-25, 1997.
- Kawabata et al., "The Fate of Plasmid DNA After Intravenous Injection in Mice: Involvement of Scavenger Receptors in Its Hepatic Uptake," *Pharm Res.*, 12: 825-830, 1995.
- Kent et al., "Enhanced T-Cell Immunogenicity and Protective Efficacy of a Human Immunodeficiency Virus Type 1 Vaccine Regimen Consisting of Consecutive Priming with DNA and Recombinant Fowlpox Virus," *J. Virol.*, 72: 10180-10188, 1998.
- Kern et al., "Target structures of the CD8(+) T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited," *J. Virol.*, 73: 8179-8184, 1999.
- Knapp et al., "A high frequency of Mamu-A\*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing," *Tissue Antigens*, 50: 657-661, 1997.
- Korber et al., "Epidemiological and Immunological Implications of the Global Variability of HIV" *Retroviral Immunology*, B. Walker, D. Pantaleo, Eds (The Humana Press, Totowa, NH, In press), 2001.
- Kuroda et al., "Analysis of Gag-specific Cytotoxic T Lymphocytes in Simian Immunodeficiency Virus-infected Rhesus Monkeys by Cell Staining with a Tetrameric Major Histocompatibility Complex Class I-Peptide Complex," *J. Exp. Med.*, 187: 1373-1381, 1998.
- Lau et al., "Cytotoxic T-cell memory without antigen," *Nature*, 369: 648-52, 1994.
- Lechner et al., "Analysis of Successful Immune Responses in Persons Infected with Hepatitis C Virus," *J. Exp. Med.*, 191: 1499-1512, 2000.
- Letvin et al., "Cytotoxic T lymphocytes specific for the simian immunodeficiency virus," *Immunol. Rev.*, 170: 127-34, 1999.
- Letvin et al., "Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination," *Proc. Natl. Acad. Sci. USA*, 94: 9378-9383, 1997.
- Levy et al., "Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8 T cells," *Immunol. Today*, 17: 217-24, 1996.
- Lew et al., "Cancer Gene Therapy Using Plasmid DNA: Pharmacokinetic Study of DNA Following Injection in Mice," *Hum. Gene Ther.*, 6: 553, 1995.
- Lewis, et al., "Limited Protection from a Pathogenic Chimeric Simian-Human Immunodeficiency Virus Challenge following Immunization with Attenuated Simian Immunodeficiency Virus," *J. Virol.*, 73: 1262-70, 1999.
- Li et al., "Infection of Cynomolgus Monkeys with a Chimeric HIV-2/SIV<sub>mac</sub> Virus That Expresses the HIV-1 Envelope Glycoproteins," *J. of AIDS*, 5: 639-646, 1992.
- Lifson et al., "The Extent of Early Viral Replication Is a Critical Determinant of the Natural History of Simian Immunodeficiency Virus Infection," *J. Virol.*, 71: 9508-14, 1997.
- Livingston et al., "The Induction of Mucosal Immunity in the Female Genital Tract Using Gene-Gun Technology (Part 1: Antigen Expression)," *Ann. New York Acad. Sci.*, 772: 265-267, 1995.
- Lu et al., "SIV DNA vaccine trial in macaques: post-challenge necropsy in vaccine and control groups," *Vaccine* 15: 920-923, 1997.
- Maecker et al., "DNA vaccination with cytokine fusion constructs biases the immune response to ovalbumin," *Vaccine*, 15: 1687-1696, 1997.
- Maecker et al., "Cytotoxic T Cell Responses to DNA Vaccination: Dependence on Antigen Presentation via Class II MHC," *J. Immunol.*, 161: 6532-6536, 1998.
- Mahnel et al., "[Experiences with immunization against orthopox viruses of humans and animals using vaccine strain MVA]," *Berl. Munch Tierarztl Wochenschr*, 107: 253-256, 1994. [English Translation of Abstract Attached].
- Manthorpe et al., "Gene Therapy by Intramuscular Injection of Plasmid DNA: Studies on Firefly Luciferase Gene Expression in Mice," *Hum. Gene Therapy*, 4: 419-431, 1993.
- Mayr et al., "[The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl).]" *Zentralbl. Bakteriol.*, 167: 375-390, 1978. [English Translation of Abstract Attached].
- McCluskie et al., "Direct Gene Transfer to the Respiratory Tract of Mice with Pure Plasmid and Lipid-Formulated DNA," *Antisense Nucleic Acid Drug Dev.*, 8: 401-414, 1998.
- Mellors et al., "Prognosis in HIV-1 Infection Predicted by the Quantity of Virus in Plasma" *Science*, 272: 1167-70, 1996.
- Montefiori et al., "Neutralizing antibodies in sera from macaques infected with chimeric simian-human immunodeficiency virus containing the envelope glycoproteins of either a laboratory-adapted variant or a primary isolate of human immunodeficiency virus type 1," *J. Virol.*, 72: 3427-3431, 1998.
- Montefiori et al., "Evaluation of antiviral drugs and neutralizing antibodies to human immunodeficiency virus by a rapid and sensitive microtiter infection assay," *J. Clin. Microbiol.*, 26: 231-237, 1988.
- Montgomery et al., "Heterologous and Homologous Protection Against Influenza A by DNA Vaccination: Optimization of DNA Vectors," *DNA Cell Biol.*, 12: 777-783, 1993.
- Moore et al., "HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells," *AIDS*, 9(Suppl. A):S117-136, 1995.
- Murali-Krishna et al., "Counting Antigen-Specific CD8 T Cells: A Reevaluation of Bystander Activation during Viral Infection," *Immunity*, 8: 177-187, 1998.
- Murali-Krishna et al., "Persistence of Memory CD8T Cells in MHC Class I-Deficient Mice," *Science*, 286: 1377-81, 1999.
- Ourmanov et al., "Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV," *J. Virol.*, 74: 2740-2751, 2000.
- Ourmanov et al., "Recombinant modified vaccinia virus ankara expressing the surface gp120 of simian immunodeficiency virus (SIV) primes for a rapid neutralizing antibody response to SIV infection in macaques," *J. Virol.*, 74: 2960-2965, 2000.
- Pal et al., "Inhibition of HIV-1 Infection by the  $\beta$ -Chemokine MDC," *Science*, 278: 695-8, 1997.
- Pertmer and Robinson, "Studies on Antibody Responses Following Neonatal Immunization with Influenza Hemagglutinin DNA or Protein," *Virology*, 257: 406-414, (1999).
- Pertmer et al., "Influenza Virus Nucleoprotein-Specific Immunoglobulin G Subclass and Cytokine Responses Elicited by DNA Vaccination Are Dependent on the Route of Vector DNA Delivery," *J. Virol.*, 70: 6119-6125, 1996.
- Pertmer et al., "Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA," *Vaccine*, 13: 1427-1430, 1995.
- Poignard et al., "Neutralizing Antibodies Have Limited Effects on the Control of Established HIV-1 Infection in Vivo," *Immunity*, 10: 431-438, 1999.
- Porgador et al., "Predominant Role for Directly Transfected Dendritic Cells in Antigen Presentation to CD8+ T Cells after Gene Gun Immunization," *J. Exp. Med.*, 188: 1075-1082, 1998.
- Power et al., "A valid ELISPOT assay for enumeration of ex vivo, antigen-specific, IFN $\gamma$ -producing T cells," *J. Immunol. Methods*, 227: 99-107, 1999.
- Quinn et al., "Viral Load and Heterosexual Transmission of Human Immunodeficiency Virus Type 1," *N. Eng. J. Med.*, 342: 921-9, 2000.
- Ramshaw and Ramsey, "The prime-boost strategy: exciting prospects for improved vaccination," *Immunol. Today*, 21: 163-5, 2000.
- Reimann et al., "An *env* Gene Derived from a Primary Human Immunodeficiency Virus Type 1 Isolate Confers High In Vivo



- Replicative Capacity to a Chimeric Simian/Human Immunodeficiency Virus in Rhesus Monkeys," *J. Virol.*, 70: 3198-3206, 1996.
- Reimann et al., "A Chimeric Simian/Human Immunodeficiency Virus Expressing a Primary Patient Human Immunodeficiency Virus Type 1 Isolate *env* Causes an AIDS-Like Disease after In Vivo Passage in Rhesus Monkeys," *J. Virol.*, 70: 6922-6928, 1996.
- Richmond et al., "Studies of the Neutralizing Activity and Avidity of Anti-Human Immunodeficiency Virus Type 1 Env Antibody Elicited by DNA Priming and Protein Boosting," *J. Virol.*, 72: 9092-9100, 1998.
- Robinson and Pertmer, "DNA Vaccines: Basic Studies and Applications," in *Adv. Virus Res.*, 55: 1-74, 2000.
- Robinson et al., "AIDS Vaccines: Heterologous Prime/Boost Strategies for raising Protective T Cell Responses", *AIDS Reviews*, 2:105-110, 2000.
- Robinson and Pertmer, "Nucleic Acid Immunizations," in *Current Protocols in Immunology*, (R. Coico, Ed.), vol. 1, pp. 2.14.1-2.14.19. 3 vols. John Wiley & Sons, Inc., New York, 1998.
- Robinson et al., "Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA," *Vaccine*, 11: 957-960, 1993.
- Robinson et al., "The Scientific Future of DNA for Immunization," *American Academy of Microbiology*, May 13-Jun. 2, 1996, 1997.
- Robinson et al., "Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations," *Nature Med.*, 5: 526, 1999.
- Rodriguez et al., "DNA Immunization: Ubiquitination of a Viral Protein Enhances Cytotoxic T-Lymphocyte Induction and Antiviral Protection but Abrogates Antibody Induction," *J. Virol.*, 71: 8497-8503, 1997.
- Ross et al., "C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge," *Nat. Immunology*, 1:127-131, 2000.
- Ross et al., "Enhanced Avidity Maturation of Antibody to Human Immunodeficiency Virus Envelope: DNA Vaccination with gp120-C3d Fusion Proteins," *AIDS Res. Human Retro.*, 17(a):829-835, 2001.
- Rubbert et al., "Multifactorial nature of non cytolytic CD8+ T cell-mediated suppression of HIV replication: beta-chemokine dependent and independent effects," *AIDS Res. Hum. Retroviruses* 13: 63-9, 1997.
- Sasaki et al., "Comparison of Intranasal and Intramuscular Immunization against Human Immunodeficiency Virus Type 1 with a DNA-Monophosphoryl Lipid A Adjuvant Vaccine," *Infect. Immunol.*, 66: 823-826, 1998.
- Sauter et al., "An Internalization Signal in the Simian Immunodeficiency Virus Transmembrane Protein Cytoplasmic Domain Modulates Expression of Envelope Glycoproteins on the Cell Surface," *J. Cell Biol.*, 132: 795-811, 1996.
- Schmitz et al., "Control of Viremia in Simian Immunodeficiency Virus Infection by CD8 Lymphocytes", *Science*, 283: 857-60, 1999.
- Schneider et al., "Induction of CD8 cells using heterologous prime-boost immunisation strategies", *Immunol. Rev.*, 170: 29-38, 1999.
- Schneider et al., "Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara," *Nat. Med.*, 4: 397-402, 1998.
- Scholtissek et al., "A cloning cartridge of  $\lambda$  t<sub>o</sub> to terminator," *Nucleic Acids Res.*, 15: 3185, 1987.
- Sizemore et al., "Attenuated *Shigella* as a DNA Delivery Vehicle for DNA-Mediated Immunization," *Science*, 270: 299-302, 1995.
- Sizemore et al., "Attenuated bacteria as a DNA delivery vehicle for DNA-mediated immunization," *Vaccine*, 15: 804-807, 1997.
- Staprans et al., "Simian Immunodeficiency Virus Disease Course Is Predicted by the Extent of Virus replication during Primary Infection", *J. Virol.*, 73:4829-39, 1999.
- Staprans et al., "Quantitative Methods to Monitor Viral Load in Simian Immunodeficiency Virus Infections," in *Viral Genome Methods*, K. Adolph, Ed., CRC Press, Boca Raton, FL, pp. 167-184, 1996).
- Stittelaar et al., "Protective immunity in macaques vaccinated with a modified vaccinia virus Ankara-based measles virus vaccine in the presence of passively acquired antibodies," *J. Virol.*, 74: 4236-4243, 2000.
- Subbarao et al., "Genetic variability of HIV-1," *AIDS*, 10(Suppl. A):S13-23, 1996.
- Sutcliffe, "Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322," *Cold Spring Harbor Quant. Biol.*, 43: 77-90, 1979.
- Sutter et al., "Nonreplicating vaccinia vector efficiently expresses recombinant genes," *Proc. Natl. Acad. Sci. USA*, 89: 10847-10851, 1992.
- Tang et al., "Genetic immunization is a simple method for eliciting an immune response," *Nature*, 356: 152-154, 1992.
- Thomson et al., "Delivery of Multiple CD8 Cytotoxic T Cell Epitopes by DNA Vaccination," *J. Immunol.*, 160: 1717-1723, 1998.
- Tobery et al., "Targeting of HIV 1 Antigens for Rapid Intracellular Degradation Enhances Cytotoxic T Lymphocyte (CTL) Recognition and the Induction of De Novo CTL Responses In Vivo After Immunization," *J. Exp. Med.*, 185: 909-920, 1997.
- Tomaras et al., "CD8 T cell-mediated suppressive activity inhibits HIV-1 after virus entry with kinetics indicating effects on virus gene expression", *Proc. Natl. Acad. Sci. U.S.A.*, 97:3503-8, 2000.
- Torres et al., "DNA immunization: effect of secretion of DNA-expressed hemagglutinins on antibody responses," *Vaccine*, 18: 805-814, 2000.
- Torres et al., "Differential Dependence on Target Site Tissue for Gene Gun and Intramuscular DNA Immunizations," *J. Immunol.*, 158: 4529-4532, 1997.
- Uchijima et al., "Optimization of Codon Usage of Plasmid DNA Vaccine Is Required for the Effective MHC Class I-Restricted T Cell Responses Against an Intracellular Bacterium," *J. Immunol.*, 161: 5594-5599, 1998.
- Ulmer et al., "Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein," *Science* 259: 1745-1749, 1993.
- Villinger et al., "Induction of Long-Term Protective Effects against Heterologous Challenge in SIVhu-Infected Macaques", *Virology*, 278:194-206, 2000.
- Waldrop et al., "Determination of Antigen-specific Memory/Effector CD4 T Cell Frequencies by Flow Cytometry", *J. Clin. Invest.*, 99:1739-50, 1997.
- Watson et al., "Plasma Viremia in Macaques Infected with Simian Immunodeficiency Virus: Plasma Viral Load Early in Infection Predicts Survival", *J. Virol.*, 71: 284-90, 1997.
- Wild et al., "Polyvalent vaccination against hepatitis B surface and core antigen using a dicistronic expression plasmid," *Vaccine*, 16: 353-360, 1998.
- Wolff et al., "Direct Gene Transfer into Mouse Muscle in Vivo," *Science*, 247: 1465-1468, 1990.
- Wu et al., "Deoxyribonucleic Acid Vaccines Encoding Antigens With Rapid Proteasome-Dependent Degradation Are Highly Efficient Inducers of Cytolytic T Lymphocytes," *J. Immunol.*, 159: 6037-6043, 1997.
- Wyand et al., "Protection by live, attenuated simian immunodeficiency virus against heterologous challenge," *J. Virol.*, 73: 8356-8363, 1999.
- Wyatt et al., "Marker Rescue of the Host Range Restriction Defects of Modified Vaccinia Virus Ankara," *Virology*, 251:334-42, 1998.
- Xiang et al., "Manipulation of the Immune Response to a Plasmid-Encoded Viral Antigen by Coinoculation with Plasmids Expressing Cytokines," *Immunity*, 2: 129-135, 1995.
- Yamamoto et al., "Highly sensitive qualitative and quantitative detection of reverse transcriptase activity: optimization, validation, and comparative analysis with other detection systems," *J. Virol. Methods*, 61: 135-143, 1996.
- Yang and Walker, "CD8T cells in human immunodeficiency virus type 1 pathogenesis: cytolytic and noncytolytic inhibition of viral replication," *Adv. Immunol.*, 66: 273-311, 1997.
- Zajac et al., "Viral Immune Evasion Due to Persistence of Activated T Cells Without Effector Function", *J. Exp. Med.*, 188:2205-13, 1998.
- Burton et al., "Why do we not have an HIV vaccine and how can we make one?" *Nature Med.* 4:495-498, 1998.

- Femberg et al., "AIDS vaccine models" Challenging challenge viruses *Nature Med.* 8(3):207-210, 2002.
- Letvin, N.L., "Progress in the development of an HIV-1 vaccine" *Science* 280:1875-1880, 1998.
- Markmeyer et al., "The pAX plasmids: new gene-fusion vectors for sequencing, mutagenesis and expression of proteins in *E.coli*," *Gene* 93:129-134 (1990).
- Mizuno et al., "Mutational analysis of two zinc-finger motifs in HIV type 1 nucleocapsid proteins: effects on proteolytic processing of Gag precursors and particle formation," *Aides Research and Human Retroviruses* 12(9):793-800 (1996).
- Wang et al., "Mammalian cell/vaccinia virus expression vectors with increased stability of retroviral sequences in *E. coli*; production of feline immunodeficiency virus envelope protein," *Gene* 153:197-202 (1995).
- Amara et al., "Different Patterns of Immune Responses but Similar Control of a Simian-Human Immunodeficiency Virus 89.6P Mucosal Challenge by Modified Vaccinia Virus Ankara (MVA) and DNA/MVA Vaccines," *J. Virology* 76:7625-7631 (2002).
- Jacobsen et al., "Characterization of Human Immunodeficiency Virus Type 1 Mutants with Decreased Sensitivity to Proteinase Inhibitor Ro 31-8959," *J. Virology* 206:527-534 (1995).
- Meyer et al., "Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence," *J. Gen. Virology* 72:1031-1038 (1991).
- Smith et al., "Recombinant Vaccinia Viruses as New Live Vaccines," *Biotechnology & Genetic Engineering Reviews* 2:383-407 (1984).
- Andersson et al. "Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 . . . " *J. of Biol. Chem.* 264(14):8222-8229, 1989.
- Cheonis, N., "Status Report on HIV Vaccine Development" Retrieved from internet <<http://www.thebody.com/sfaf/winter00/vaccine.html>. Oct. 2001.
- Ho, M., "AIDS Vaccines Trials Dangerous" Retrieved from the internet <http://www.i-sis.org.uk/isisnews/i-sisnews11-19.php>. Oct. 2001.
- \* cited by examiner

*Fig. 1*

1/4

1 CCGCAATATT GGCTATTGGC CATTGCATAC GTTGTAICTA TATCATATTA TGATCAITTA TATTGGCTCA TGTCGAATAT GACCGGCCATG TTGACATTGA  
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AATAACTGAT CAATAATTAAT CATTAGTTAA TGCCCAAGTA ATCAAGTATC GGGTATATAC CTCNAGGCGC AATGTATTGA ATGCCAITTA CCGGCGGAC

201 GTGACGCGC CAACGACCCC CGCCCAITGA CGTCAATTAAT GAGGTATGTT CCCATAGTAA GCCAATAGG GACTTTCCAT TGACGTCAAT GGGTGGAGTA  
CGACTGCGG GTTGCTGGG GCGGTAAT .GCACTTATTA CTGCATACAA GGGTATCATT GCGGTATCC CTGAAGGTA ACTGCAGTTA CCCACCTCAT

301 TTTACGGTAA ACTGCCCACT TGGCAGTACA TCAAGTGTAT CATATGCCAA GTCCGCCCCC TATTGACGTC AATGACGTA AATGCCCGC CTGGCATTAT  
AATGGCAATT TGACGGGTGA ACCGTCACTGT AGTTACATA GTATACGGTT CAGGCGGGG ATAACGTCAG TTAAGTCCAT TTACCGGGG GACCGTAATA

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501 GCGGTGATA GCGGTTTGAC TCACGGGGAT TTCCAGTCT CCACCCCAT GACGTCAATG GGAGTTTGT TTGGCACCAA AATCAACGGG ACTTTCCAAA  
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901 TTCCATTAGC TATAGGTGAT GGTATAGCTT AGCCTATAGG TGTGGGTTAT TGACCACTAT TGACCACTCC CCTATTGCTG ACGTACTTTC CCATTACTAA  
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1001 TCCATACAT GGCTCTTTGC CACAATATC TCTATTGGCT ATATGCCAT ACTCTGTCT TCAAGACTG ACACGGACTC TGTATTTTA CAGGATGGG  
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1101 TCCGATTTAT TATTTACAA TTCACATATA CACACACCG GTCCCGCTG CCGGAGTTT TTATTAACA TAGCGTGGG TCTCCACGG AATCTCGGT  
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1201 ACGTGTCCG GACATGGGCT CTCTCCGGT ACGGGCGGAG CTTCCACATC CGAGCCCTG TCCCATGCTT CAGGCGCTC ATGCTGCTC GGCAGTCTCT  
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Fig. 2A

2/4

1301	TGCTCTTAAC AGTGGAGGCC AGACTTAGGC ACAGACAAT GCCCACCAC ACCAGTGTGC CGCACAGGC CGTGGGGTA GGTATGTGT CTGAAATGA ACGAGGATTG TCACCTCCGG TCTGAATCCG TGTGCTGTTA CGGGTGGTGG TGGTCACAGC GCSTGTTCCG GCACGGCCAT CCCATACACA GACTTTTACT
1401	GCTCGGAGAT TGGGCTCGCA CCGCTGAGCG AGATGGAGA CTTAAGGCAG CGGCAGAGA AGATCAGGC AGCTGAGTTG TTGTATTCTG ATAGAGTCA CGAGCTCTA ACCCGAGCGT GCGGACTGCG TCTACCTTCT GAATCCGTC GCGCTTCT TCTAGTCCG TCGACTCAAC AACATAAGAC TATTCTCAGT
1501	GAGTAACATC CCGTTGCGGT GCTGTTAAG GTGGAGGGA GTGATCTG AGCATCTC GTTGTGCG CGGGCGCCAC CAGACATAAT AGTCCACAGA CTCCATTGAG GGCACGCCA CGACATTCG CACCTCCCGT CACATCTGAG TCGTCATGAG CAACGACGC GCGGCGGTG GTCTGTATTA TCGACTGTCT
1601	CTAACAGACT GTTCTTTCC ATGGTCTTT TCTGCAGTCA CCATCGATGC TTGCATCAT GCATGCATG AACAGAGGC TCTGCTGTGT GCTGCTGTG GATTGTCTGA CAAGGAAAG TACCAGAAA AGACGTGAGT GGTAGTACG AACGTTAGTA CTTCTCTCCG AGACGACACA CGACGACGAC
1701	TGTGGAGCAG TCTTCTGTTT GGTAGCCCG GGTGATAAA CGACCGCGC ARTCCCTAGG CTGTGCTTC TAGTTGCCAG CCATCTGTTG TTTGCCCTTC ACACCTCGTC AGAAGCAAG CCGATCGGG CCCACTATT GCTTGGCGG TTAGGATCC GACACGGAAG ATCAACGGTC GGTAGACAAC AACCGGGAG
1801	CCCCGTGCT TCTTGAACC TGGAGGTGC CACTCCOACT GTCTTTCCT AATAAATGA GGAATTCGA TCGCATTCG TGAGTAGGTG TCATTCTATT GGGGCAGGA AGGACTGGG ACCTTCCAG GTAGGGTGA CAGGAAGGA TTATTTTACT CCTTACGT ACCGTAACAG ACTCATCCAC AGTAAGATAA
1901	CTGGGGTG GGTGGGGA GCACAGCAG GGGGAGGATT GGGAGACAA TAGCAGGCAT GCTGGGAG GCTGGGGTGC TATATAAAA ACGCCGGG GACCCCCAC CCACCCCGT CCTGTCGTT CCGTCTTAA CCTTCTGT ATGTCGTA CGACCCCTAC GCCACCCGAG ATATATTTT TCGGGGCGC
2001	GCAACCGAG GTTCTGAAC CTAGAGTGA CAATTCAGA AGAATCTGC ARAGAGCGA TAGAAGCGA TCGCTGCGA ATCGGGAGCG GCGATACCGT CGTTGGCTCG CAAGACTTC GATCTCAGT GTTAAGTCT TCTTGAAGT TCTTCCGCT ATCTCCGCT ACAGACCGT TAGCCTCGC CGCTATGGCA
2101	AAAGCAGAG GAAGCGTCA GCCATTGC GCCAAGTC TTCAGCAATA TCACGGGTAG CCAACGCTAT GTCTGTATG CCGTCTGCA CACCCAGCGG TTTCGTGCTC CTTGCCAGT CCGGTAGCG GCGGTGAG AGTCTGAT AGTCCCATC GTTGGGATA CAGGACTATC GCCAGACGT GTGGTCCGC
2201	GCACAGTGC ATGAATCCAG AAAGGGGC ATTTCCACC ATGATATCG GCAAGAGG ATCGGCAAGG GTACAGAGA GATCTCGC GTCGGGCATG CGGTGTGAG TACTTAGGT TTTGCGCG TAAAGGTGG TACTATAGC CGTCTCCG TAGCGGTACC CAGTCTGCT CTAGGAGCG CAGCCCGTAC

Fig. 2B

3/4

2301	CTGCGCTTGA GCCTGGCGAA CAGTTCGGCT GGCGGAGCC CCTGATGTC TTGCTCAGA TCATCTGAT CGACAGACC GGCTTCCATC CGAGTACGTG GAGCGNACT CGGACCGGTT GTCAAGCGA CCGCGCTGG GACTACAG AGCAGGCTT AGTAGACTA GCTGTCTGG CCGAAGGTAG GCTCATGCAC
2401	CTGCTCGAT GCGATGTTTC GCTTGGTGGT CGAATGGGA GGTAGCCGA TCAACGGAT GCAGCGCGG CATTCATCA GCCATGATGG ATACTTCTC GAGCGAGCTA CGCTACAAAG CGAACCACTA GCTTACCGT CCATCGGCT AGTCCGATA GCTCGCGGC GTAACGTAGT CGGTACTACC TATGAAGAG
2501	GGCAGGAGCA AGGTGAGATG ACAGGAGATC CTGCCCCGGC ACTTCCCGCA ATAGAGCCA GTCCCTTCCC GCTTCAGTGA CAAGTCCGAG CACAGCTGGC CCGTCTCGT TCCACTCTAC TGTCTCTAG GACGGGCGG TGAAGCGGT TATCTCGGT CAGGGAAGG CGAAGTCACT GTTGCAGTCT GTTGCACCG
2601	CAAGGAAGCC CCGTCTGGC CAGCCACGAT AGCCGCGGTG COTCGCTTC CAGTTCATTC AGGGCACCG ACAGTCCGT CTTGACAAA AGAACGGGC GTTCCCTTGG GGCAGACCG GTCGGTGCTA TCGGGCGGAC GGAGCAGAAC GTCAAGTAA TCCCGTGGC TGTCACCCA GAAGTGTCTT TCTTGGCCG
2701	GGCCCTGGC TGACAGCGG AACACGGCG CATCAGAGCA GCGGATGTC TGTGTGCC AGCTATAGCC GAATAGGCTC TCCACCCAG CGGCCGAGG CGGGACCG ACTGTCGGC TTGTGCGGC GTAGTCTCGT CGGCTAACAG ACAACAGGG CTAGTATCGG CTTATCGGAG AGGTGGGTTC GCGGCTCT
2801	ACCTGGGTG ANTCCATCTT GTTCATCAT GGAACGAT CCTATCCG TCTTTGATC AGATCTTAT CCGCTGGCC ATCAGATCCT TGGCGGCAAG TGGACGACG TTAGGTAGAA CAAGTAGTA CGCTTGTCTA GAGTAGGAC AGAGACTAG TCTAGRACTA GGGGACCGG TAGTCTAGA ACCGCGTTC
2901	AAAGCATCC AGTTTACTTT GCAGGGCTTC CCAACCTTAC CAGAGGCGC CCAGCTGGC AATCCGGTT CGCTGCTGT CCATAAAACC GCCAGTCTA TTTCGGTAG TCAATGAAA CGTCCGGAAG GTTCGGAATG GTCTCCGGG GGTGACCG TTAAGCCAA GGTATTTGG CCGGTTCAGT
3001	GCTATCGCA TGTAAAGCCA CTGCAAGCTA COTGGTTCT CTTCGGCTT GGGTTTCCC TTGTCAGAT AGCCAGTAG CTGACATTC TCCGGGGTCA CGATAGCGGT ACATTGCGGT GACGTTGAT GCACGAAGA GAACCGGA CCGAAGGG ACAGGTCTA TCGGGTCATC GACTGTAAGT AGGCCCACT
3101	GCACGTTTC TCGGACTGG CTTTCTACGT GAAGGATC TAGTGAAGA TCTTTTGA TAATCTCATG ACCAAATCC CTTAACGTGA GTTTCTGTT CGTGGCAAG ACGCCTGACC GAAAGATGCA CTTTCTCTAG ATCCACTTCT AGGAAACT AGGAAACT ATTAGATAC TGGTTTTAGG GAATGCACT CAAAGCAAG
3201	CACGAGGT CAGACCCGT AGAAGATC AAGGATCTT CTTGATCC TTTTCTTG CCGGTATCT GCTGCTTGA AACAAAANA CCACCGCTAC GTGACTCGCA GTCTGGGCA TCTTTCTAG TTCTTAGAA GAATCTAGG AAAAAAGAC GGCATTAGA CGACGAAGT TTGTTTTTTT GGTGGCGATG
3301	CAGCGTGT TTGTTTGGC GATCAGAGC TACCACTCT TTTTCCGAAG GTAACGTGCT TCCAGCAGC GCAGATACCA AATCTGTTC TTCTAGTGA GTGCGCACCA AACAAAGGC CTAGTCTCG ATGGTTGAGA AAGGCTTC CATTGACCGA AGTGTCTCG CGTCTATGGT TTATGACAG AAGATCACAT
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3501	TGCTTACCG GGTGGACTC AAGACATAG TTACCGGATA AGGCGAGC GTGGGCTGA ACGGGGGTT CTTGACACA GCCCAGCTG GAGCGAAGCA ACAGAAATGC CCAACCTGAG TTCTGTATC AATGGCTAT TCCGCTGCG CAGCCGACT TGCCCCCAA GCAGTGTGT CCGGTGGAAC CTCGCTTGT

Fig. 2C

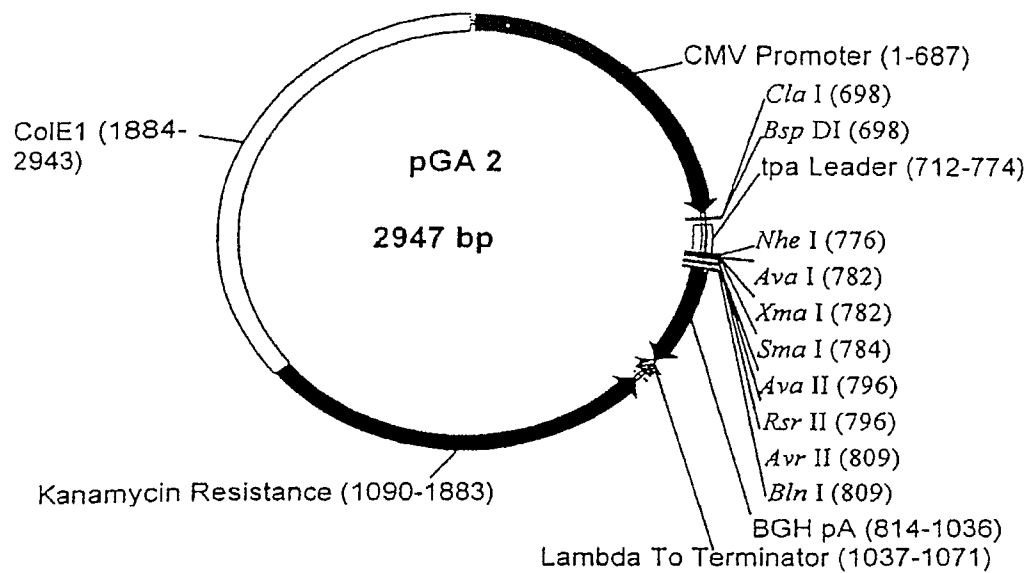
4/4

3601 CCTACACCGA ACTGAGATAC CTACAGCGTG AGCTATGAGA AAGGCCACG CTTCCGAAG GGAGAAAGC GGACAGGTAT CCGGTAAGC GCAGGTCGG  
GGATGTGGCT TGACTCTATG GATGTCCAC TCGATACTCT TTCGGGTGC GAAGGGCTC CCTCTTTCCG CCGTCCATA GGCCATTCCG CGTCCAGCC  
Cole1

3701 AACAGGAG CGCAGGAGG AGCTTCCAGG GGGAACGCC TGGTATCTTT ATAGTCCTGT CGGTTTCCG CACCTCTGAC TTGAGCGTCG ATTTTGTGA  
TTGTCCTCTC GCGTGCTCC TCGAAGGTCC CCCTTGGG ACCATAGAAA TATCAGGACA GCCCAAGCG GTGGAGACTG AACTCGCAGC TAAACACT  
Cole1

3801 TGCTCGTCAG GGGGGGGAG CCTATGGAAA AACCCAGCA AGCGGCCCT TTTACGGTTC CTGGCCTTTT GCTGGCCTTT TGCTCACATG TTGT  
ACGAGCAGTC CCCCCCCTC GGATACCTTT TTGGGCTCGT TCGGCGGGA AATGCCAAG GACCGGAAA CGACCGGAAA ACGAGTGTAC AACA  
Cole1

*Fig. 2D*

*Fig. 3*



1	CGGAAATATT GCGTATTGGC CATTGCAATC GTTCTATCTA TATCATATAA TGTGGCTCA TGCCCAATAT GACCGCCATG TTGACATTGA -GCTTTTAA CCGATAACCG GTAAGGTATG CACATAGAT ATACATATTAT ACATGATAT ATACCGGAT ACAGTTTATA CTGGCGGTAC AACTGTAACT
101	TTATGACATA GTTATTAATA GTATCATATT ACGGGTCAT TTGTTCTATG CCGATATATG GAGTTCGGG TTACATTAAT TACGTAATAT GGCCTGGCTG ATPACTGAT CANTATATAT CATTTAGTTAA TGCCCGAGTA ATCAAGATATC GGTATATATC CTCAGAGCG ATGTATATGA ATGCCATTTA CCGCGCGGAC
201	GCTGCGCCCG CAACGACCC CCGCCATTTGA GGTCAATAT GAGTATGTT CCGATATGTA CCGCATATAG GCTTTTCCAT TGCATGCAAT GGTGGAGTA CGACTGCGCG GTTGCTGGGG CCGGCTAACT CAGTTTATA CCGCATACA GGTATCATTT GCGGTATATC CTGAAGGTA ATGCAATTA CCGACTCAT
301	TTTACGGTAA ACTGCCACT TGGCATGTA TCAAGTGTAT CATATGCCAA GTCCGCCCC TATTGAGTC ATGACGGTA ATGCGCCCG CTGGCATTA AAATGCCATT TGACGGGTGA ACCGTCACTG AGTTACATA GTATACGTT CAGCGCGCG ATACTGCG TTACTGCCAT TTACCGGCG GACCTAATA
401	GCCGATGCA TGACCTTAGG GCACTTTGCT ACTTGGCAT ACTTGGCAT ACTTGGCAT ACTTGGCAT ACTTGGCAT ACTTGGCAT ACTTGGCAT ACTTGGCAT CGGTCATGT ACTGATATG CTTGAAAGGA TGAACGGTCA TGTAGATGCA TATATCATAG CANTATATGT ACCACTAGCG CANAACGTC ATGTGGTTAC
501	GCGGTGGATA GCGGTTTAC TCACGGGGAT TTCCAGTCT CACCCCATTT GAGTCTATG GAGTTTGT TTGGACCAA ATCAACGGG ACTTTCCAA CCGCACTAT CCGCAACTG ATGCGCCCTA AAGTTTCA GGTGGGTAA CTGAGTTAC CTTCAACAA ACCGTTGTT TTAGTTGCC TGAAGGTTT
601	ATGTCGTAT AACCGCCCG CATTGACGA ATGCGCGCT AGCGGTATC GGTGGGAGT CTATATAAG AGAGTGTGTT TAGTGACATC ATTCATATCA TACAGCATTA TTGGGGGGGG GCACTGCGT TTACCGGCA TTGCGCATG CCACTCTCA GATATATG TCTGAGCAA ATCACTTAG TAGATAGCT
701	TGCTTCGAT CATGATGCA ATGAGAGAG GGTCTGCTG TGTGCTGCTG CTGTGTGAG CAGTTTCTG TTGCGTAGC CCGGGTAT ANACGCGCG ACGAGGTGA GTACTAGGT TACTTCTCT CCGAGACAC ACACGACAC GACACACTC GTGAGAGCA AGCGGATCG GCGCCCTCTA TTGCTGCG
801	H D A H K R G L C C V L L C G A V F V S Avc II tpa leader
901	CCGATCCT AGGCTGTGCT TCTAGTTGC CAGCATCTG TTGTTGGCC CTCCCGCTG CTTCTCTCA CCTTGGAGG TGCACTCC ACTGTCTTT CGCTTAGGA TCCGACCGG AAGATCAACG GTGCTGAC ACACACCGG GAGGGGAC GAGAGGACT GGGCTTCC AGGTGAGG TGACAGGAA
1001	CCTATATAA TGAGGAATT GATGCGAT GTCTGAGTAG GTCTCTCTT ATTTGCGGG GTGGGTGGG CAGAGACAGC AAGGGGAGG ATTGGAGAA GGATTATTT ACTCTTTAA CAGATCATC CAGATCATC CAGATCATC CAGATCATC CAGATCATC CAGATCATC CAGATCATC CAGATCATC CAGATCATC
1101	CATACAGG CATGCTGGG ATCGGTGGG CTCTATATAA AAGCGCCCG GCGGACCG AGCTTTCTA AGCTTATG CAGACATTC AGAGAACTC GTATCTGCC GTACGACCC TAGCCACCC GAGATATAT TTTCGGGCG CCGCTTGGC TCGCAGACT TCGCATCTCA CTTGTTTAA TCTTTTGG
1201	GTCHAGAGG CGATAGAGG CGATGCGCTG CGATGCGGA GCGGCTATC GGTATGAGG CAGAGAGCG TACGCCAT CCGCGGCACT CTCTTACGA CAGTTCTTCC GCTATCTCC GCTACGGAC GCTTAGCCT CCGGCTATG GATTTCTG CTCTTGGC AGTGGGTAA CCGGGGTC GAGAGTGT
	ATATCAGGG TAGCCACCC TATGTCTGA TACGGGTG CACACCCCG CCGGACCG TGTATGATC CAGAAAGCG GCAATTTCC ACCATGAT TATGTGCC ATCGGTTGG ATACAGACT ATCGGACAG GGTGCGGTG GCGGCTGTC AGCTGATG AGCTACTTAG GTCTTTTCC CCGTAAAGG TGCTACTATA

Fig. 4A

1301	TCGGCAAGCA GGCATCGCCA TGCGTCACGA CGAGATCGTC CCGCTCGGCG ATGCTCGCTT TGAGCTGCGG GACAGCTTCG GCTTGGCGGA GCGCCTGATG AGCCGTTGCT CCGTAGCGGT ACCAGTGCT GCTTAGAGCG CGGACGCGCG TAGAGGACCG ACTCGAGCG CTTGTCAAGC GACCCGCT CGGGGACTAC
1401	CTCTTCCTCC ACATCATCTT GATCGACAG ACCGCTCTCC ATCCGAGTAC GTGCTGCTTC GATCGGATGT TTGCTGTTGT GTGCTGATGG GCGAGTAGCC GAGAGCAGG TCTAGTAGGA CTAGCTGTC TGCGCGAGG TAGGCTCATG CAGAGCGGCG CTAGCTTACA AGCGACCA CCGCTTACC CGTCCATCG
1501	GGATCAAGCG TATGCGAGCG CGGANTTCCA TCAGGCTATG TGGATCTTT CTGCGGAGA GCGAGGTGAG ATGACAGGAG ATCTCGCCCG GCGACTTCGG CCTAGTTGCG ATAGCTGCGC GCGCTAACGT AGTCCGTACT ACCTATGAAA GACCGCTCTT CTTTCACTC TACTGTCTC TAGGCGGGG CCGTGAAGCG
1601	CCAAATAGCAG CCGATCCCTT CCGGCTTCAG TGACAAAGTC GACACAGCT GCGTAGGGA CCGCGCTGCT GGTACGCCAC GATAGCGCG CTGCTCTCTC GCTTATCGTC GGTCAAGGAA GGGCGAAGTC ACTGTTGCGA CCGCTTCTT CCGGCGAGCA CCGGTGCGTC CTATCGGCGC GACGCGACAG
1701	TTGCACTTCA TTACGGGCGAC CGGACAGGTC GGTCTTGACA AAAGAGCG GCGGCGCTTG CGCTGACAGC CGGACAGCG CGGACATCAGA GCGCGGAT AACGTCAAGT AAGTCCGCGT GCTTCCGAG CCGACTCTT TTCTTGGC CGCGCGGAC GCGACTGTG CCGTGTGCG CCGCTAGTCT CCGTGGCTAA
1801	GTCTGTTGTG CCGAGTCATA GCGGATAGC CTCTCCACC ACGCGCGCG AGACCTGCG TCGATCTGCG TCGATCTGCG TCGATCTGCG GATCTCTATC CAGACAACAC GGGTCACTAT CCGCTTAGCG GACGCTGCG TTGCGCGCG TCTGAGCGC ACCTTAGGTA GAACAAGTAA GTAGGCTTTG CTAGGACTG
1901	CTGCTCTTTG ATCAGATCTT GATCCCTGCG GCGATCAGT CTTTGGCGCG AAGAAAGCA TCCAGTTTAC TTTCGAGGCG TTCCACCTT TACCAAGCG GACAGAGAC TAGCTTAGAA CTAGGGAGCG CCGTAGTCTA GGAACGCGCG TTCTTGGT AGCTCAGTC MACGTCCCG AAGGCTTGA ATGCTCTCC
2001	GCGCCAGCT GGAATTCG GTTGGCTGCG TGTCCTTACA ACCGCGCT GTAGCTATG CCGCTGACG CCGCTGACG CCGCTGACG CCGCTGACG CCGCTGACG GCGGGTGA CCGTTAGGCG CAGCGCAGCG ACAGTATTT TGGCGGTC GATCGATGCG GGTAGCTTC GGTAGCTTC GGTAGCTTC GGTAGCTTC GGTAGCTTC
2101	CTTCCGTTT CCGTTGTCCA GATAGCCGAG TAGCTGACAT TCACTCGGCG TAGACAGCT TTCTGCGGAG TGCTTTCTTA GGTGMAAGG ATCTAGTGA GACGCGAAA GGGACAGCT CTATCGGCTC ATCGACTGTA ATAGGCGCG ACCTGCGGA AAGCGGCTG ACCTGCGGA AAGCGGCTG ACCTGCGGA
2201	AGATCCCTTT TCAATATCTC ATGACGAAA TCCCTTAGG TGATTTTGG TTCCACTGAG CCGTACAGCG CCGTACAGCG CCGTACAGCG CCGTACAGCG TCTAGGAAA ACTATAGAG TACTGCTTT AGGGAATTC ACTAAAGC AAGTGAAGT GCGCTGCGG GCGCTGCGG GCGCTGCGG GCGCTGCGG GCGCTGCGG
2301	TCCTTTT TTTTCTGTTT GCAACAAA AAGCCAGCG TACAGGCTT TCGGCTGCGG AAGTTCGCA AAGTTCGCA AAGTTCGCA AAGTTCGCA AAGTTCGCA AGGAAAAA GACCGCAT AGACGCGAA CTTTGTGTTT TTTGCGCGG AAGTTCGCA AAGTTCGCA AAGTTCGCA AAGTTCGCA AAGTTCGCA AAGTTCGCA
2401	AAGTAACTG GCTTACGAG AGCGGATA CCAATATG TTCTTCTAG TTAGCGCTAG TTAGCGCTAG TTAGCGCTAG TTAGCGCTAG TTAGCGCTAG TTAGCGCTAG TTCCATTGAC CCAAGTCTG TCGGCTCTAT GCTTATGAC AAGAGATCA CACCGGCTC AATCCGCTG TGAAGTCTT GAGACATCT GCGGATGTA
2501	ACCTCGCTCT GCTAATCTG TTACAGTGG CTGCTGCGAG TGCGATAG TCTGCTCTTA CCGGCTTGA CCGGCTTGA CCGGCTTGA CCGGCTTGA CCGGCTTGA TGGAGCCAGA CCGTTAGGAC AATGCTCAG GACGAGGTC ACCGCTATC ACCGCTATC ACCGCTATC ACCGCTATC ACCGCTATC ACCGCTATC ACCGCTATC
2601	GCGGTGCGGC TGACGCGGG GTTCTGAC ACAGCCGAG TTGGAGGGA CCGCTACAC CCGCTACAC CCGCTACAC CCGCTACAC CCGCTACAC CCGCTACAC CGCCAGCCCG ACTTGCCTCC CAGCAGCTG TGTGCGGCTG AACTGCTT GCTGATGCT GCTGATGCT GCTGATGCT GCTGATGCT GCTGATGCT GCTGATGCT
2701	ACGCTTCCG AAGGAGAAA GCGGACAGG TATCCGCTTA GCGGAGCT GCGGACAGA GCGGACAGA GCGGACAGA GCGGACAGA GCGGACAGA GCGGACAGA TCCGAGGCG TTCCCTCTT CCGCTGCTC ATAGGCTAT CCGCTGCTC CCGCTGCTC CCGCTGCTC CCGCTGCTC CCGCTGCTC CCGCTGCTC CCGCTGCTC
2801	TTTATAGTC TGTGCGGTTT CCGCACCTCT GACTTAGCG TCGATCTCT CAGGCGGCG GAGCTATG AAGAGGCG AAGAGGCG AAGAGGCG AAGAGGCG AAATATCAG ACGACCCAAA CCGGTGAGGA GGAATCTG ACCTAAGAC ACTAGGAGA GTCCCCCG CTGCGTACC TTTTCCGCT GTTTCGCGG

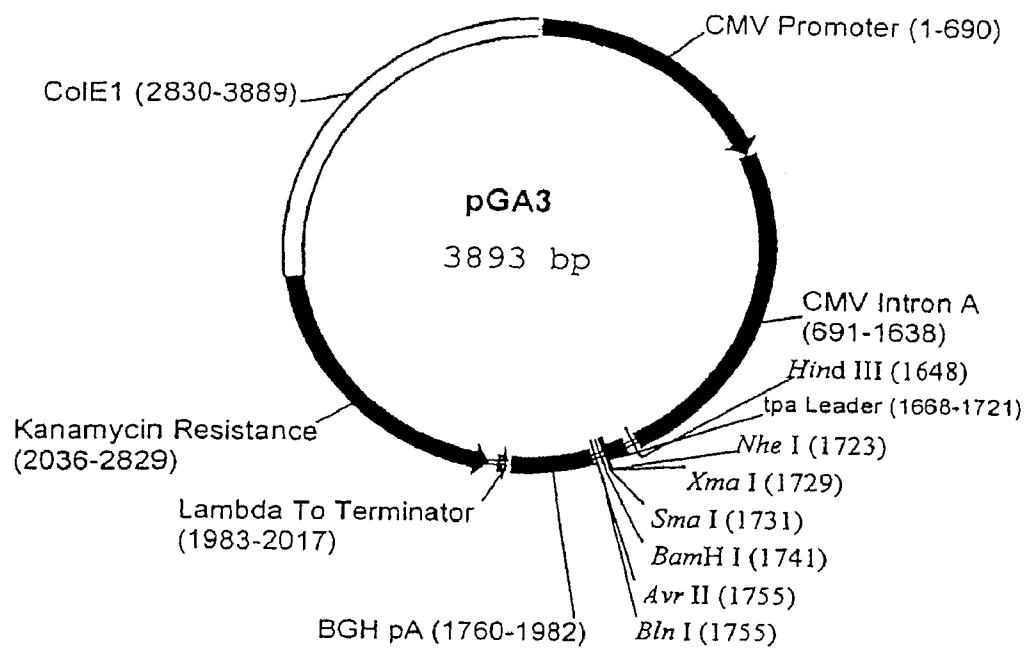
Fig. 4B

3/3

2901 CCTTTTACGG TTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTTGT  
GGAAAATGCC AAGGACCGGA AAACGACCGG AAAACGAGTG TACAACA  
ColE1

---

***Fig. 4C***

*Fig. 5*

1	CGACAATTT GCGTATTGGC CATTTGCATAC GTTGTATCTA TATCATATAA TGACATTTTA TATTGGCTCA TGTCCTATAT GACCGCCATG TTGACATTGA GCTGTTATRA CGGATNACCG GTTACGTTATG CACATATAGT ATAGTATTTT ACAGTTAAAT ATATACCGAGT ACAGTTATA CTGGCGGTAC TACTGTAACT
101	TTATTGACTA GTTATTAAATA GTAATCAATT ACGGTTTCAT TAGTTCTATG CCGATATAG GAGTTCCGGG TTACATNACT TACGGTAAAT GCGCCGCGTG AATAACTGAT CAATTAATTAT CATTTAGTTTA TCCCAAGTA ATCAAGTATC GGSTATATAC CTCAGGCGG AATGTATTGA ATGCCATTTA CCGGCGGAC
201	GCTGACCGCC CAACGACGCC CCGCCATGA COTCAATAT GAGCTGTT CCGATAGTAA GCGCATATAG GACTTTCCAT TGACGTCAAT GGGTGGAGTA CGACTGGCGG GTTGTCTGGG GGGGCTRACT CCACTTATTA CTGCATACAA GGGTATCAAT CCGTTATACC CTGAAGGTA ACTGCAGTTA CCCACTCAT
301	TTTACGGTAA ACTGCCCACT TGGCAGTACA TCACGTGTAT CATATGCCAA GTCCGCCCC TATTGAGCTC AATGACGGTA AATGCCCCG CTGGCATAT AANTGCCATT TGACGGGTGA ACCGTCACTG AGTTACATA GTATACGGTT CAGCGGGGG ATAACTGCAG TTACTGCCAT TTACCGGGCG GACCGTAAAT
401	GCCAGTACA TGACCTTACG GCACTTTCCCT ACTTGGCAGT ACATCTACT ATTAGTCATC GCTATTACCA TGGTGATGCG GTTTTGGCAG TACACCAATG CGGGTCATGT ACTGCAATGC COTGAAAGGA TGAAACGGTCA TGTAGATGCA TAATCAGTAS CCAATATGCT ACCACTAGCG CAAAACCGTC ATGTGCTTAC
501	GCGTGCGATA GCGGTTTGAC TCACGGGGAT TTCCAGTCT CCACCCCAT GACGTCTATG GGGTTTGGT TTGGCACCAA AATCAACGGG ACTTCCAAA CGCCACTAT CCCCNACTG AGTGCCTCTA AAGTTTCA GAATGGTTA CTGCAGTTAC CTTCAACAAA AACCGTGGT TTAGTTGCC TCAAGAGTTT
601	ATGTGCTAAT AACCCCGCCC CGTTGAGGCA AATGGGGGT AGGCTGTAC GTGGGAGGT CTNTATAGC AGACTGCTT TATGTAAACCG TCAGATGCGC TACAGCATTA TTGGGGCGGG GCACTGGCT TTACCGGCA TCAGACATG CACCCCTCCA GATATATTG TCTGAGCAA ATCACTTGGC AGCTAGCGG
701	TGGAGAGGCC ATCCAGCGTG TTTTGACCTC CATGAGAC ACCGGACCG ATCCAGCTC GCGCGCGGG AACGGTGCAT TGAACCGCG ATTCCCGGTG ACCTCTCGGG TAGGTGGAC AATAGTGAG GTATCTCTG TGCGCTTGGC TAGTGGGAG GCGCGCGCC TTGOCACGTA ACCTTGGCGC TANGGGGAC
801	CCAAGATGA CGTAGTACC GCTATAGC TCTATAGGA CACCCCTTGG GCTCTATGC ATGCTATACT GTTTTGGCT TGGGGCCCTAT ACACCCCGC GTTTCTACT GCATTCATGG CCGATATCTG AGATATCCGT GTGGGGAAC CGAGATPAG TACGATATGA CAAAACCGA ACCCGCGATA TGTGGGGCG
901	TTCCCTTAGC TATAGTGAT GGTATAGCTT AGCCTATAGG TGTGGTTAT TGACCTTAT TGACCACTCC CTTATTGGTG ACGTACTTT CCATTACTAA AAGGNATPAG ATATCCACTA CCAATATCGAA TCGGATATCC AATCCCAATA TATACGGTTA TGAGACAGGA AGTCTCTGAC TCTGCTGAG ACATAAATAT GTCTATGAT
1001	TCCATAACAT GGTCTTTGC CACAACTATC TCTATTGGCT ATATGCCAAT ACTCTGTCT TCAAGAGACTG ACACGGACTC TGTATTTTAA CAGGATGGG AGGTATTCTA CCGAGAAAGC GTGTTGATAG AGATAACCGA CMT Intron A
1101	TCCCATTTAT TATTACAAA TTCAATATA CAACAAGCC GTCCCGCTG CCGCAGTTT TTATTAAACA TAGGCTGGGA TCTCCACGG NATCTCGGT AGGTAATAA ATAAATGTTT AAGTGTATAT GTTGTTCGG CMT Intron A
1201	ACGTGTTCCG GACATGGGCT CTTCTCCGGT AGCGCGGAG CTTCCATC GAGCCCTGG TCCATNGCT CCAGCGACTC ATGGTGCCTC GGCAGTCTCT TGACAAAGGC CTGTACCGA GAAGAGGCA TCGCGGCTC GAAGGTAG CTTCCGAGC AGGTACGGA GTTCGGCGAG TACCAGCGAG CCGTCGAGGA
1301	TGCTCTTAA CAGTGGAGCC AGCTTAGGC ACAGCAAT GGCACACCC ACCAGTGGC GGCACAGGC CCGTGGCGTA GGGTATGTGT CTGAATATGA ACGAGGATTG TCACCTCGG TCTGAATCCG TGTCTGTTA CCGGTGGTGG TGTGACAGS CCGTGTTCGG GCACCGCAT CCCATACACA GACTTTTACT
1401	GCTCGAGAT TGGCTTCGCA CCGGTGAGC AGATGGAGA CTTAAGCAG CCGGAGAGA AGATGACAGG AGTGAAGTTG TTGTATTCTG ATAGAGTCA CGAGCTCTA ACCCGAGCGT GCGCACTGCG TCTACCTCT GATTCGCTC CCGCTCTCT TCTACGTCCG TCGACTCAAC AACATNAGAC TATTCACAT
	CMT Intron A

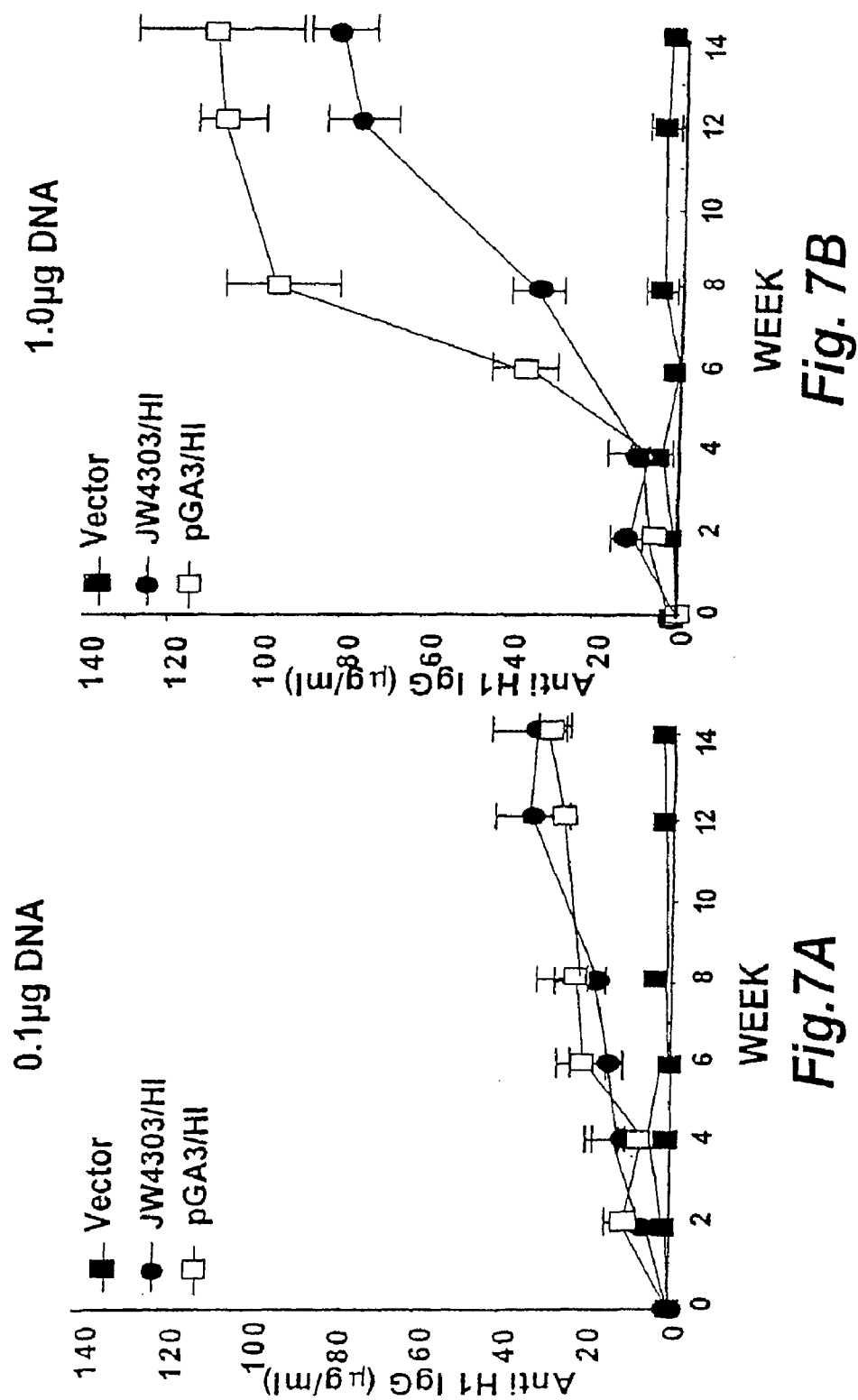
Fig. 6A

**Fig. 6B**

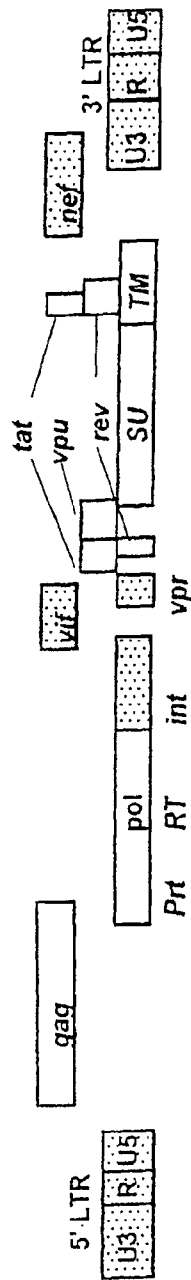
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2701	CCCCGCGCT GACAGCCGGA ACACGGCGGC ATCAGAGCAG CCGATTGCTT GTTGGCCCA GTCATAGCCG AATAGCTCTT CCACCCNAGC GGCCGGAGAA GGGACGCGA CTGTGCGCT TGTCCCGCG TAGTCTGTC GGTACACAGA CAACACGGT CAGTATCGGC TTATCGGAGA GGTGGGTTCG CCGGCTCTT
2801	CTTGCTGCA ATCCATCTTG TTCAATCATG CGAACCATC CTATCCCTGT CTCTTGATCA GATCTTGATC CCCTGGCCA TCAGATCTTT GGCGCAAGA GGACCACTT TAGGTAGAAC AAGTTAGTAC GCTTTGCTAG GAGTAGACA GAGAACTACT CTAGAACTAGT GGGACGCGGT AGTCTAGGAA CCGCCGTTCT
2901	AGCCATCCA GTTTACTTTG CAGGCTTCC CACCTTACC AGAGGGGCC CGAGGTGGCA ATTCGGTTC GCTTGTGTC CATAAACCG CCCAGTCTAG TTCCGTAGGT CAATGAAAC GTCCGGAAG GTTGAATGG TCTCCCGCG GGTGACCGT TAAGGCCAG CGAACGACAG GTATTTTGGC GGTTCAGATC
3001	CTATGCCAT GTAGCCCAAC TGCAAGCTAC CTGCTTTCTC TTTGCGCTTG CTTTTTCCCT TGTCAGATA GCCAGTAGC TGCAATTCAI CCGGGGTGAG GATAGCGTA CATTCGGGTG ACGTTGCTG GACGAAGAG AAGCGGAAC GCAAAAGGA ACAGGTCTAT CCGGTCTATG ACTGTAAGTA GGCCCCAGTC
3101	CACCGTTTCT GCGGACTGCC TTTCTACGTG AARAGGATCT AGGTAGAT CTCTTTTGAT AATCTCATGA CCAAAATCC TTAACTGAG TTTTCTTCC GTGGCAAGA CCGCTGACG AAGATGCAC TTTTCTTAGA TCCACTCTA GGAATACTA TTAGAGTACT GGTTTTAGG AATTGCATC AAAGCAAGG
3201	ACTGAGGTC AGACCCGTA GAAAGATCA AAGATCTTC TTGAGATCTT TTTTCTGCG GGTAACTCG CTGCTTGCAA ACAAAAAC CACCGCTACC TGACTGCCAG TCTGGGGCAT CTTTTCTAGT TTCTAGAG AACTCTAGGA AAAAAGAGC GCATTAGAC GACGAACGTT TGTTTTTG GTGGCGATCG
3301	ACGGGTGTT TGTTCGCG TAGTCTCGA TGGTTGAGA AAGAGCTTC TTTCGAGG TAACGGCTT CAGCAGAGC CAGATACCAA ATACTGTTCT TCTACTGTAG TGGCCACCAA ACNACGGCC TAGTCTCGA TGGTTGAGA AAGAGCTTC ATTGACCGAA GTCTCTCCG GTCTATGGT TATGACAAGA AGATCAGATC
3401	CGGTAGTAG GCCACACTT CAAGAATCT GTAGCAGCG CTACATACCT CGCTCTGCTA ATCTGTTAC CAGTGGCTGC TGCCAGTGGC GATAGTCTG GCCATCAATC CCGTGGTGA GTTCTTGA CATCTGGCG GATGTATGGA GCGAGACGAT TAGGACAATG GTCCCGAGC ACGGTACCG CTATTCAGCA
3501	GTCTTACCG GTTGGACTA AGCAGTAGT TACCGGTAA GCGCAGCGG TCGGGCTGAA CCGGGGGTTC GTGCACACAG CCCAGCTTG AGCGAACGAC CAGAAATGGC CAACCTGAGT TCTGCTATCA ATGCCCTATT CCGCTCGCC AGCCCGACTT GCGCCCCAAG CACGTGTGTC GGTTCGAACC TCGCTTGCTG
3601	CTACACGGA CTGAGATCC TACAGCTGA GCTATGAA AGCCGACGC TTCCCGAAGS GAGAAAGGCG GNCAGGTATC CGGTAAAGCG CAGGTTCGGA GATGTGGCTT GACTCTATGG ATGTGCACT CGATACTCTT TCGGGGTGCG AAGGGCTTCC TCTTTCCCG CTGTCCATAG GCCATTTCGCC GTCCCGCCT
3701	ACAGGAGAG GCACGAGGA GCTTCCAGG GAAACGCTT GGTATCTTAA TAGTCTGTC GGGTTTCGCC ACCTCTGACT TCAGGCTGCA TTTTGTGAT TGTCTCTCG CGTGCTCCCT CGAAGTCCC CTTTGGGA CCATAGAAAT ATCAGGACAG CCCAAGCGG TGGAGACTGA ACTCGCAGCT AAAACACTA
3801	GCTCGTCAGG GGGGGGAGC CTATGGAANA ACGCCAGCA CCGGGCCCTT TTACGGTTC TGGCTTTTG CTGGCTTTT GCTCACATGT TGT CGAGCAGTCC CCCCCTCG GATACCTTTT TCGGGTCTGT GCGCGGGA ATGCGAAG ACCGGAAC GACCGAANA CAGGTGTACA ACA

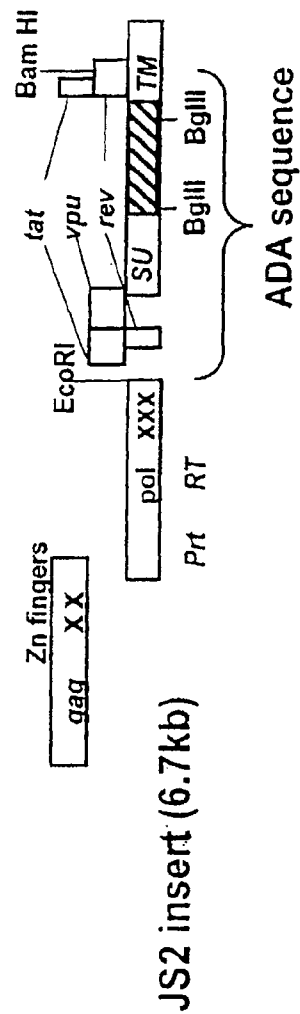
Fig. 6C



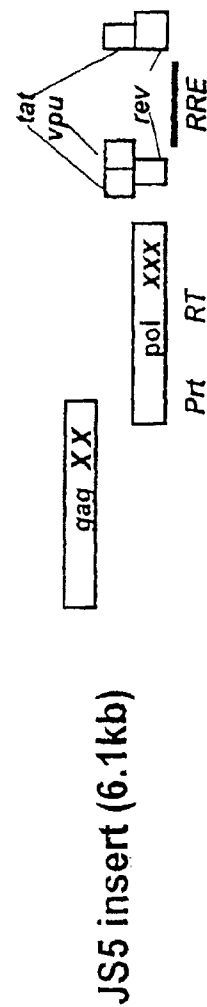




**Fig. 8A**



**Fig. 8B**



**Fig. 8C**

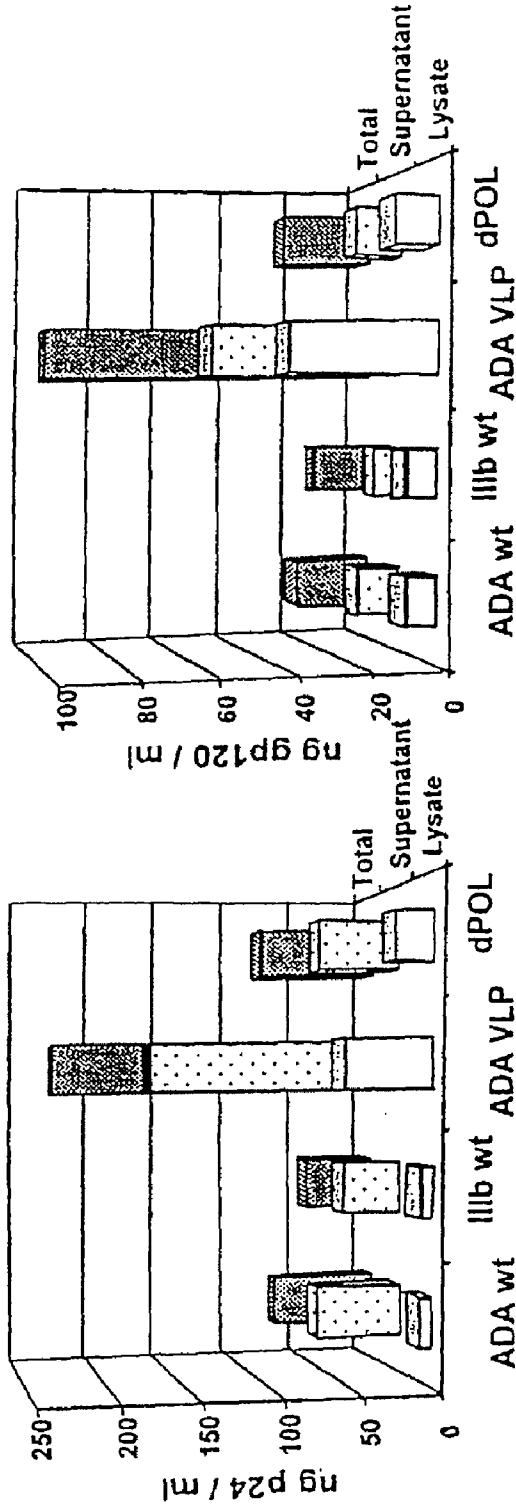


Fig.9A

Fig.9B

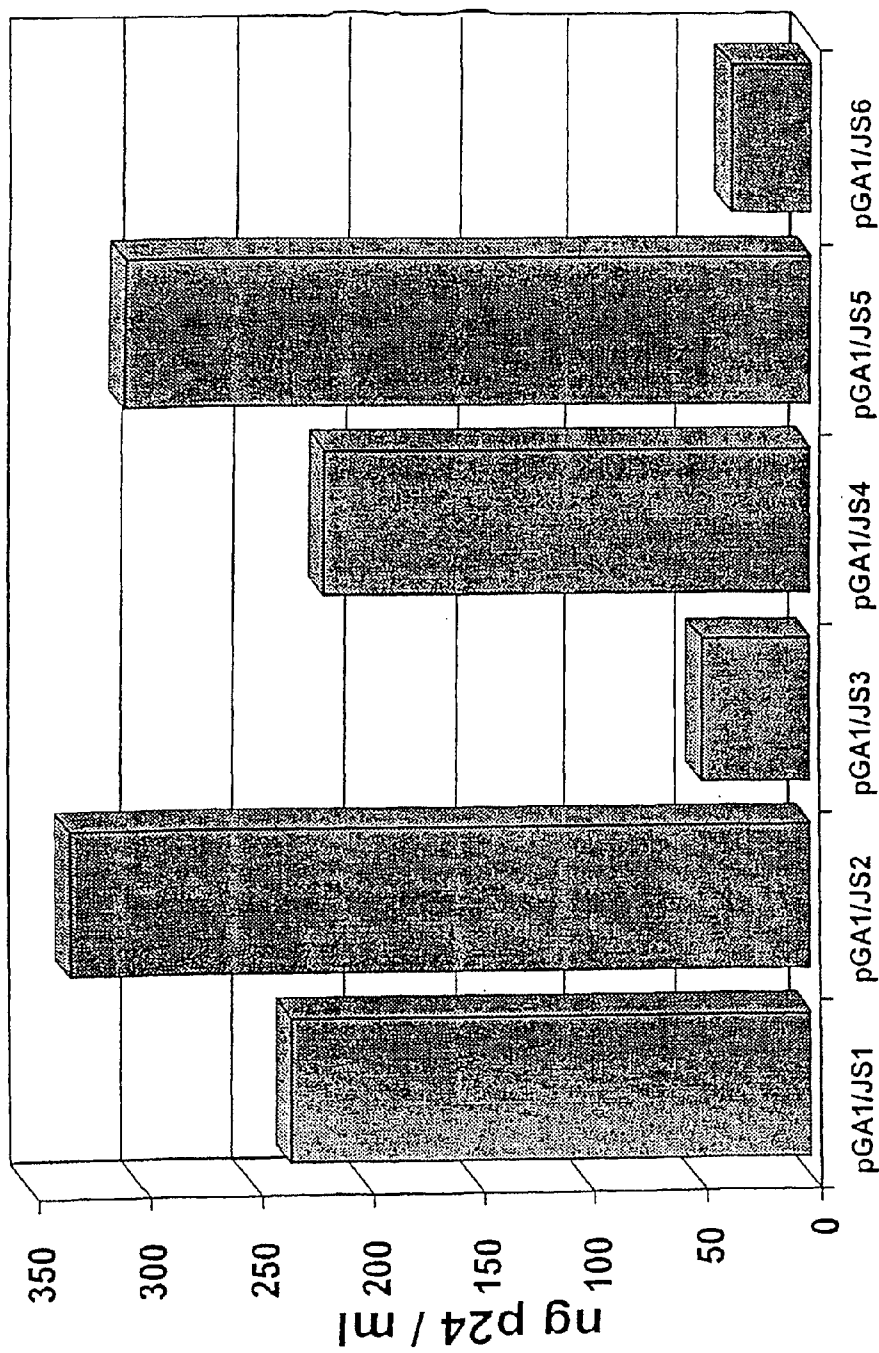


Fig. 10

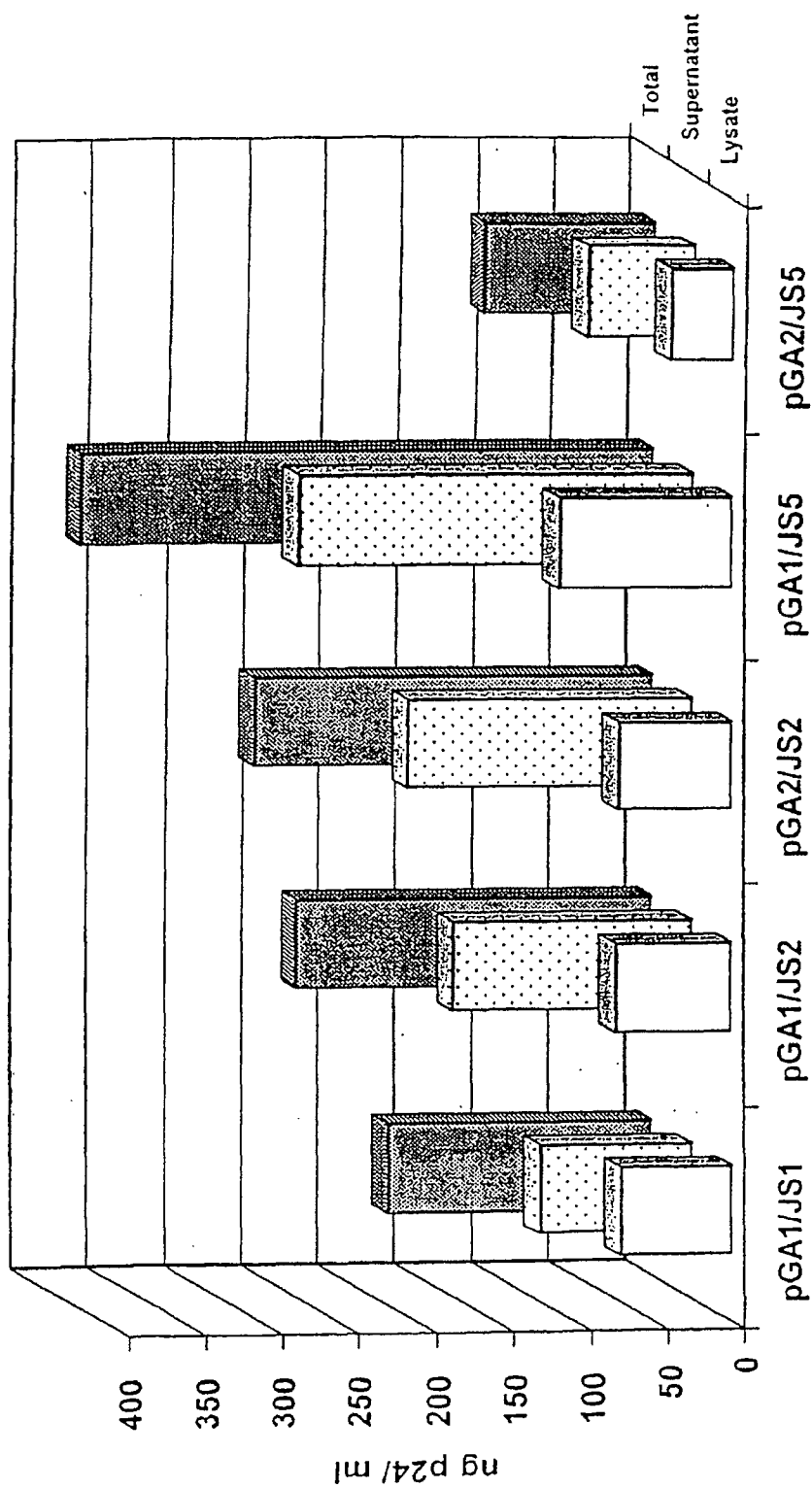


Fig.11A

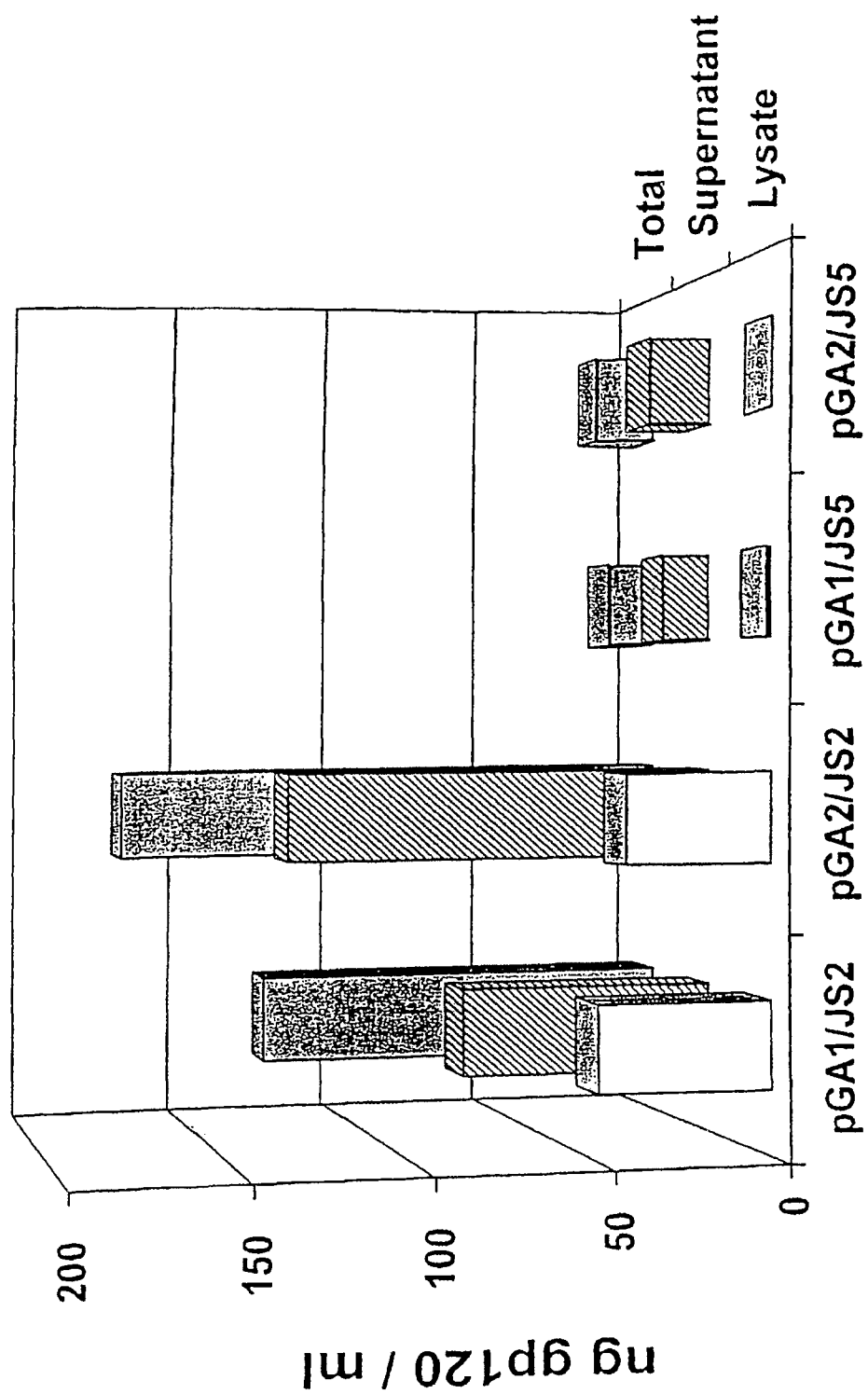


Fig.11B



Fig.12A



Fig.12C

Fig.12B

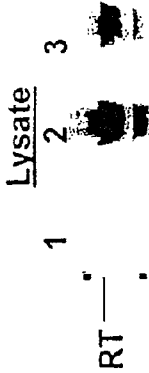
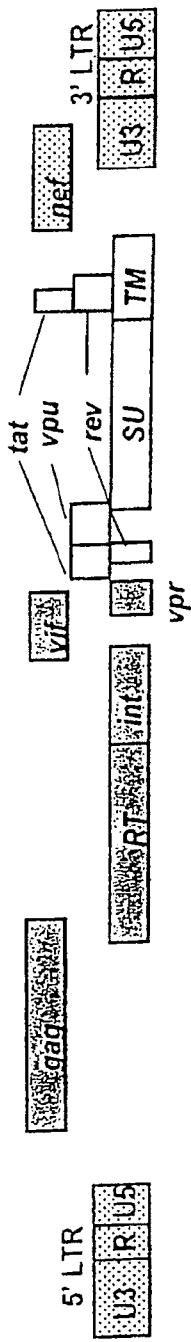
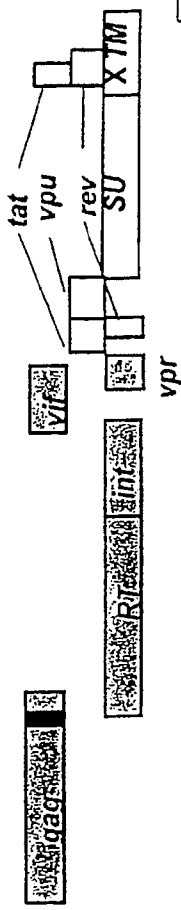


Fig.12D

SHIV 89.6



pGA2/89.6



pGA1/Gag-Pol

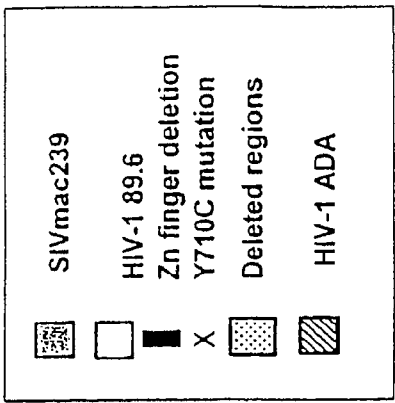
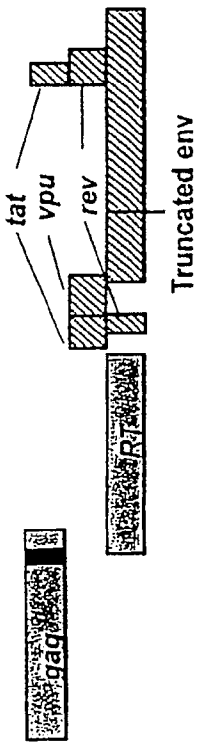
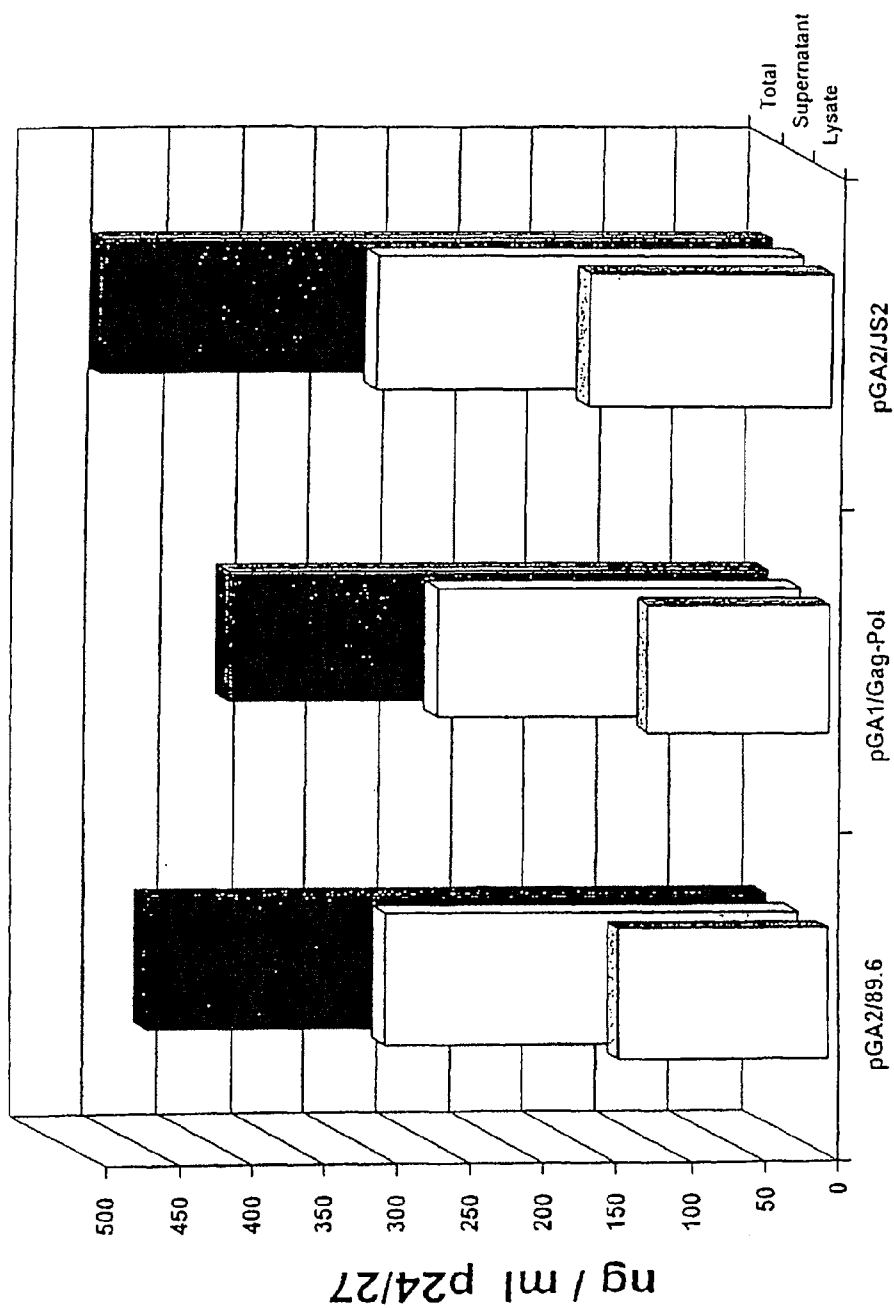


Fig.13



**Fig.14**



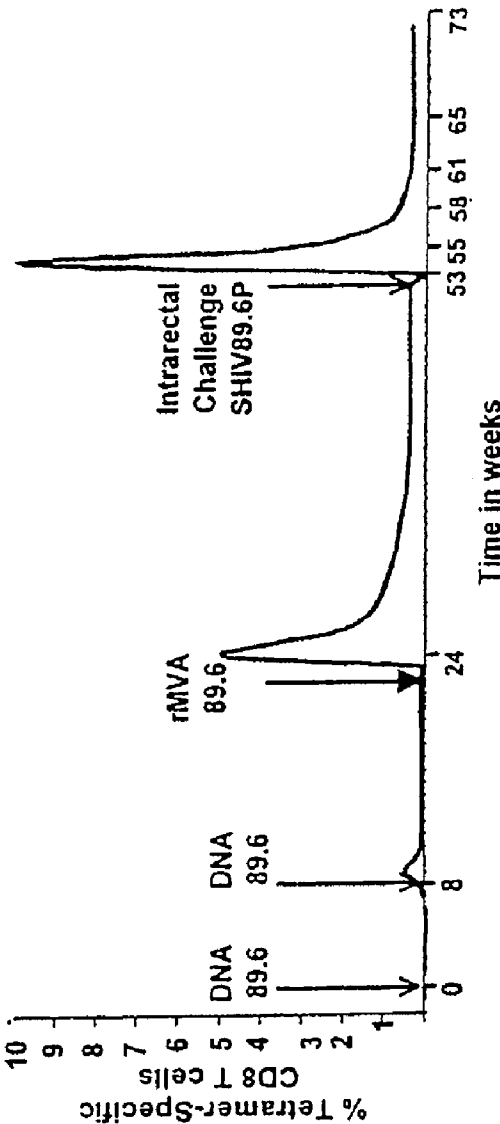


Fig. 15A

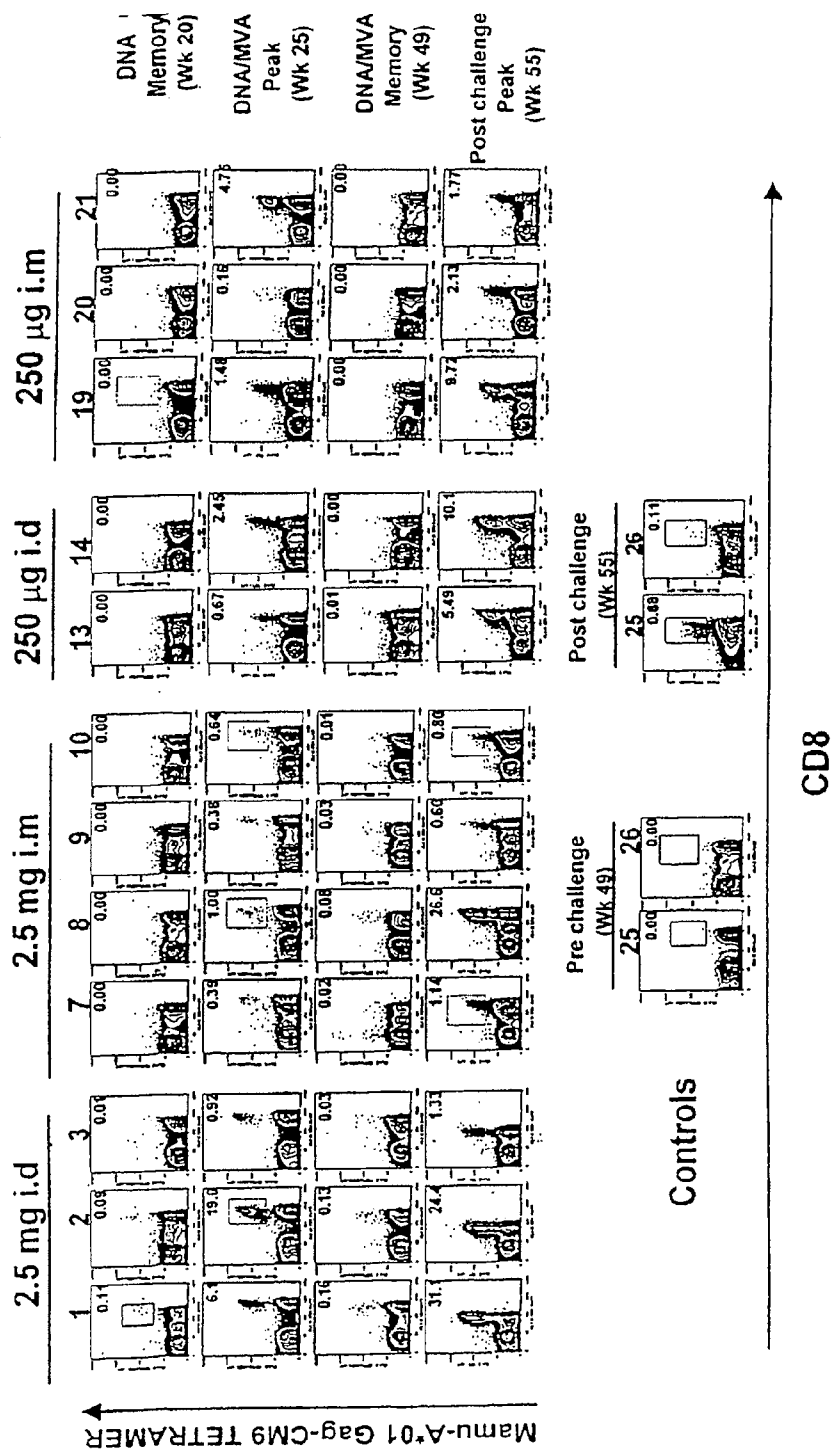


Fig.15B

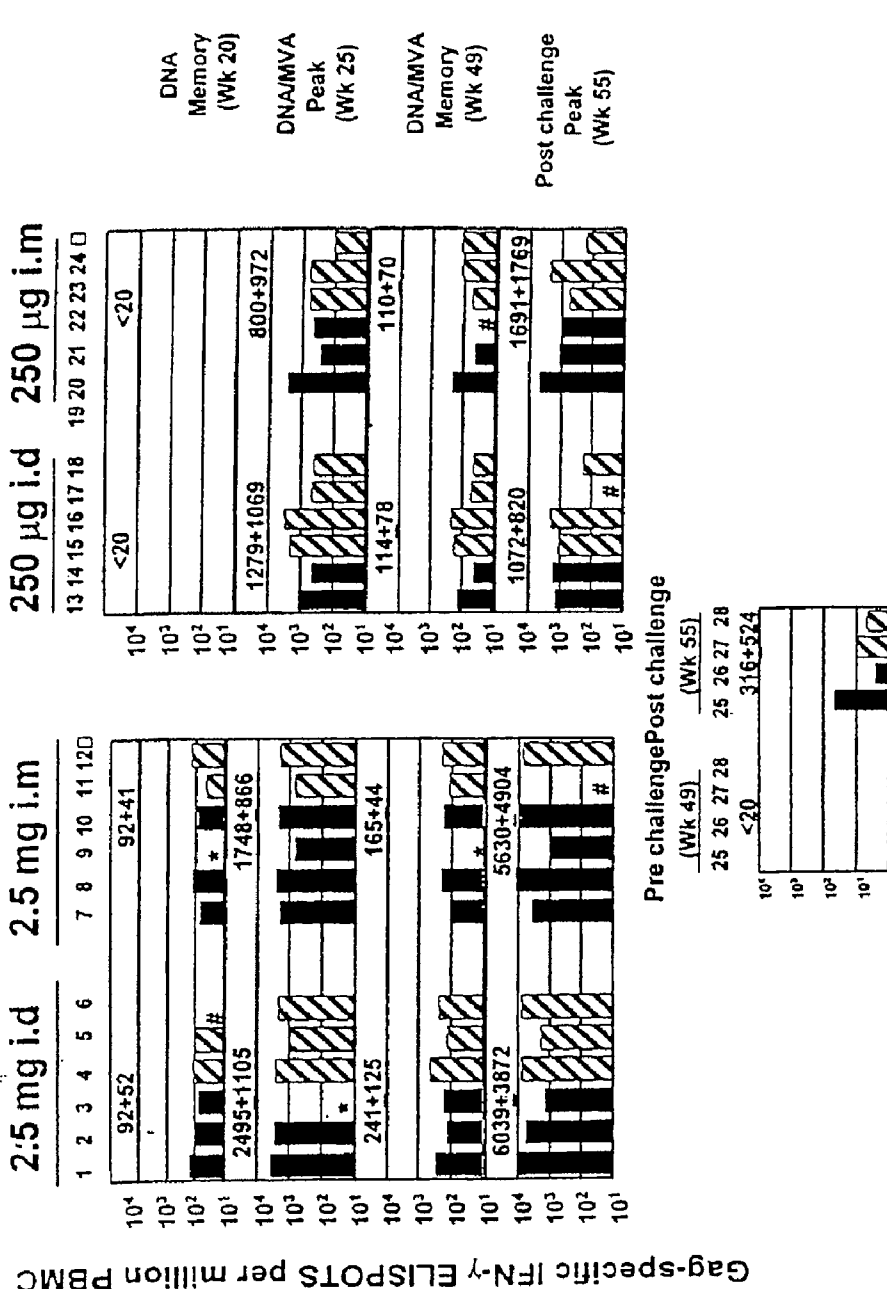
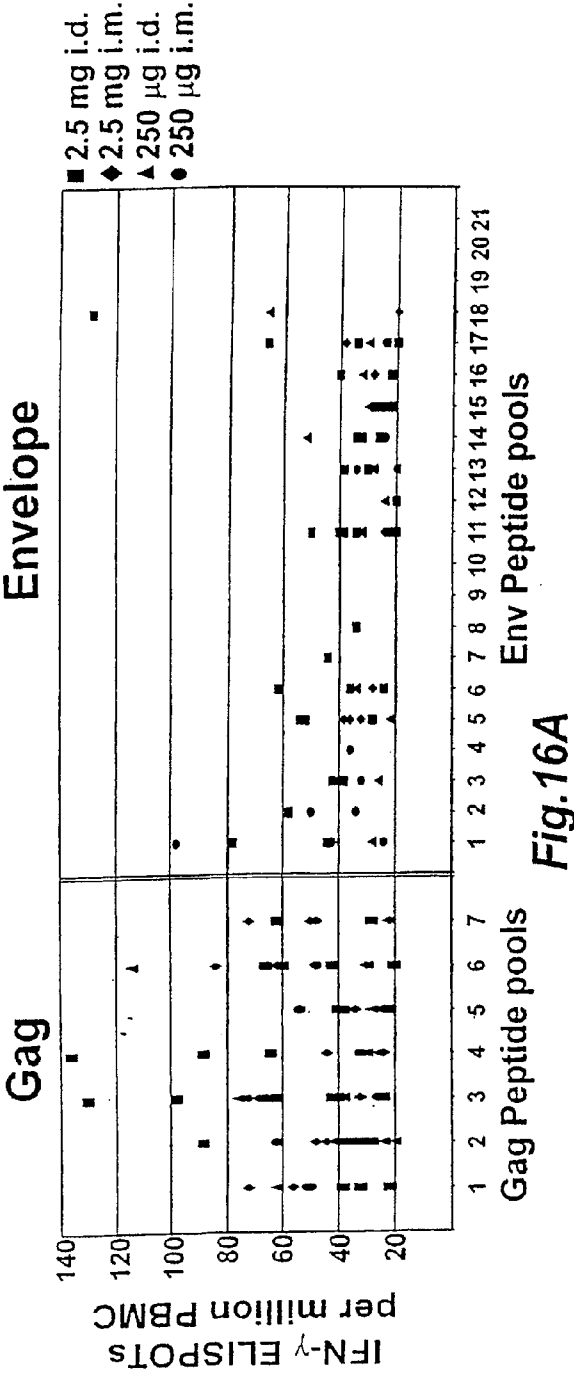


Fig.15C



DNA Prime	Height of ELISPOTs			Breadth of ELISPOTs		
	Gag	Env	Total	Gag	Env	Total
2.5 mg, i.d.	285 +130	224 +76	509 +137	4.5+1.2	5.3+2.0	9.8+2.4
2.5 mg, i.m.	203 +97	83+44	286 +73	4.4+1.8	2.8+1.3	7.2+1.1
250 $\mu$ g, i.d.	104 +113	101 +77	205 +183	2.2+2.5	3.5+2.4	5.7+4.5
250 $\mu$ g, i.m.	76 +99	84 +110	160 +207	1.7+2.0	2.3+2.9	4.0+4.7
Control DNA	0	0	0	0	0	0

**Fig. 16B**

**Fig. 17A**



3/6

AAATAGACAGGTTAATTGATNGGTAACACAGAAAGACAGACAGTGGC	4200
ATGAAAGTGAAGGGGATCAGAGAAATATCAGCACTCTGCGAAATGGGGCATCTGCTCTTGGGATGTTGATGATCTGTACTCTGTAGAAATTTG	
tat	
rev	
M K V K G I R K N Y Q H L W K W G I H L L G H L H I C S A V E N L	
ADA env	
TGGGTACAGTTTATTATGGGTACCTGTGTGGAAAGAGCAACCAACCACTCTATTTTGTGCATCAGATGCTTAAGCATATGATACAGAGGTACTATATGTTTGGGCCACACATGCTGTGTACCCACAGACCCCAACCCACAGAGTA	4350
tat	
rev	
W V T V Y Y G V P V W K E A T T T L F C A S D A K A Y D T E V H N V W A T H A C V P T D P N P Q E V	
ADA env	
GTTTGGAAATGTGACAGAAATTTTACATGTGGAAATATACATGTTAGACAGATGCCATGAGGATATATCAGTTTATGGGATCAAGCCATGTAAGCCATGTGTAAATTAACCCCACTCTGTGTACTTTAAATTCACACTGATTG	4500
tat	
rev	
V L E N V T E N F N H W K N N H V E Q H H E D I I S L W D Q S L K P C V K L T P L C V T L N C T D L	
ADA env	
AGGAATGTACTAATATCATATAGTAGTGGGGAATGAGAGGAAATATAAARACTTGCTCTTTCAATATCACACAGCATAGAGGTAAAGTGAAGAGACACTATGCCACTTTTTRTAGACTGTGTAGTACCAATGATAATGAT	4650
tat	
rev	
R N V T N I N N S S E G H R G E I K N C S F N I T T S I R D K V K K D Y A L F Y R L D V V P I D N D	
ADA env	
AATACTAGCTATAGGTTGATAAATTTGTAATACCTCAACCATATACAGGGCCGTGTCUAAAGGTATCTTTGACCCATTCCTCATATTTATGTACCCCGCTGTTTTGGGATTTCTTAAAGTGTAAAGACAGAGANGTTCAATGGACAGGG	4800
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rev	
N T S Y R L I N C N T S T I T Q A C P K V S F E P I P I H Y C T P A G F A I L K C K D K K F N G T G	
ADA env	
CCATGTAAATGTGACAGAGTACAATGTACACATGGAATAGCCGCTAGTGTCTCACTCACTGCTGTAAATGGCGAGTCTAGCAGAGAGAGGTAGTAAATTTAGTAAATTTTACAGAGACATGCAAAAACATAATAGTACG	4950
tat	
rev	
P C K N V S T V Q C T H G I R P V V S T Q L L L N G S L A E E E V V I R S S N F T D N A K N I I V Q	
ADA env	
TTGGAAGATCTGTAGAAATTAATTGTACAGAGCCACACACATACAGGAAAGTATACATATAGCCAGGAGGAGGATTTTATACACAGAGAGAAATATAGGAGATRTAGACAGACATTCGACATTTAGTAGACAAATGG	5100
tat	
rev	

Fig. 17C

4/6	
L K E S V E I N C T R P N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C N I S R T K W	
ADA env	5250
ATAACACTTTAAATCAATAGTACAAAATTAAAGACACAAATTTGGGAAATTAATAAACAAATAGTCTTTAATCAATCTCAGAGGGGACCCAGAAATTTGTATCCACAGTTTAAATTTGGAGGGCAATTTTCTACCTGTAATTCACAA	
tac	
rev	
N H T L N Q I A T K L K E Q E G N N K T I V F N Q S S G G D P E I V H S F N C G G E F E Y C M S T	
ADA env	5900
CARCTGTTTAATAGTACTTGGAAATTTACACAAATCCAAATGCTAGCAAGCAATGCTAGCAATTAACAACAATTTATTAATATGTCAGGAGTAGGAAAGCAATGTATCCCT	
tac	
rev	
Q L F H S T W N E N G T W N L T Q S N G T E G N D T I T I L P C R I K Q I I N H W Q E V G K A H Y A P	
ADA env	5550
CCCATCAGAGGACAAATTAGCTCATCAATATTACAGGCTAATATTGGGGAGAGATATGAGGCAATTCGACAGTGAATTAATAATAACAAGAGATGGTGGAACTAGTGGTCCGAGATCTTCAGACCTG	
tac	
rev	
P I R G Q I R C S N I T G L I L T R D G T N S S G S E I F R P G G G D M R D N W R S E L Y K Y K	
ADA env	5700
GTAGTAAATTTGACCATTAGGATAGCACCCACCAAGCCAAAGAGAGAGTGTGCGAGAGAAAGACAGCCAGTGGAGACCATAGGACTATGCTTGGTTTGGAGCAGCAGCACTATGGCCGACGCTCAATACG	
tac	
rev	
V V K I E P L C V R P T K A K R R V V Q R E K R A V G T I G A H F L G F L G A A G S T H G A A S I T	
ADA env	5850
CTGACGGTACAGGCCAGACTATATTGTCTGGTATAGTCACAGCAGACAAATTTGCTGAGGCTATTGAGGCCCAACAGCATCTTGCACTCAGCATCTGGGCAATCAGCAGCTCCAGCAGAGCTCTGGCTTCGAAGCATRC	
tac	
rev	
L T V Q A R L L L S G I V Q Q N N L L R A I E A O Q H L L Q L T V W G I K Q L Q A R V L A L E R Y	
ADA env	6000
CTAGGGATCAACAGCTCTAGGGATTTGGGTTGCTCTGGAAATCTCATCTGCGCCACTCTGCTGCTGGATGCTTGGGCTGATTAATAACTCTGGATATGATTTGGATTAACATGACCTGGATGGAGTGGGAAGAGAAATCGAA	
tac	
rev	
L R D Q Q L L G I W G C S G K L I C T T A V P W N A S W S N K T L D H I W D N H T W M E W E R E I E	
ADA env	

Fig. 17D





[illegible]

**Fig. 17F**

[illegible]

**Fig. 18A**





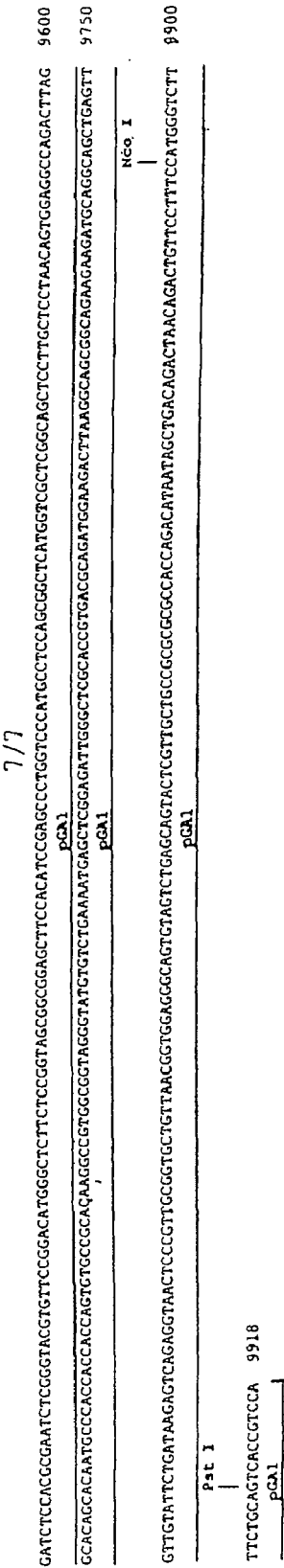


[illegible]

**Fig. 18E**







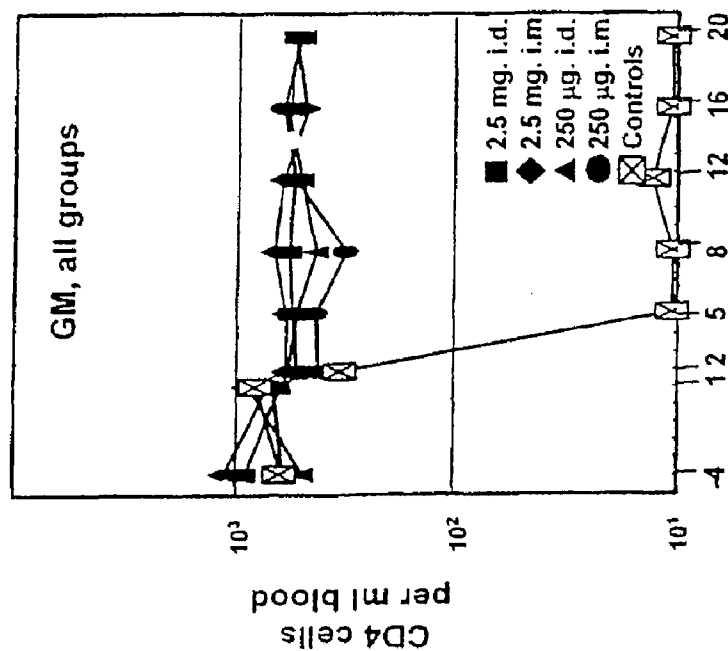


Fig. 19B

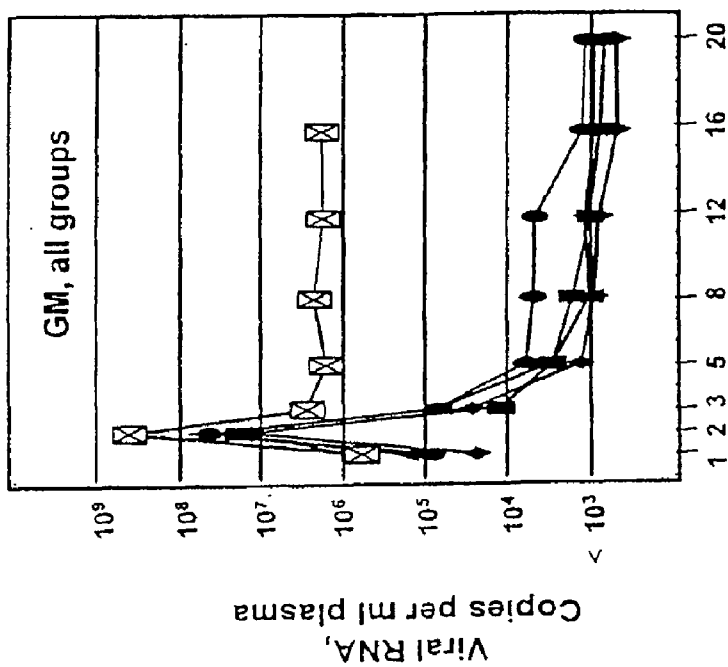
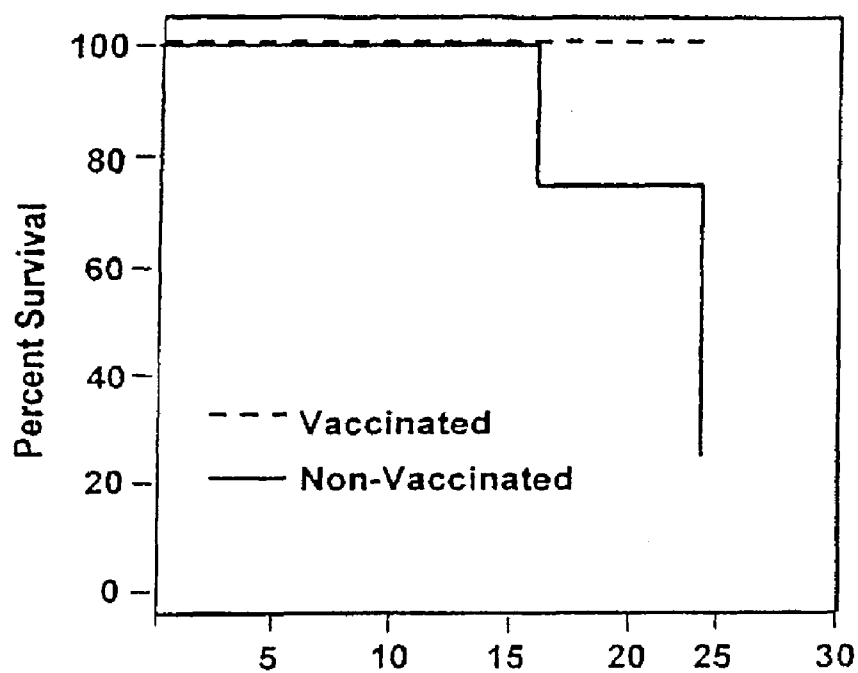


Fig. 19A

*Fig.19C*

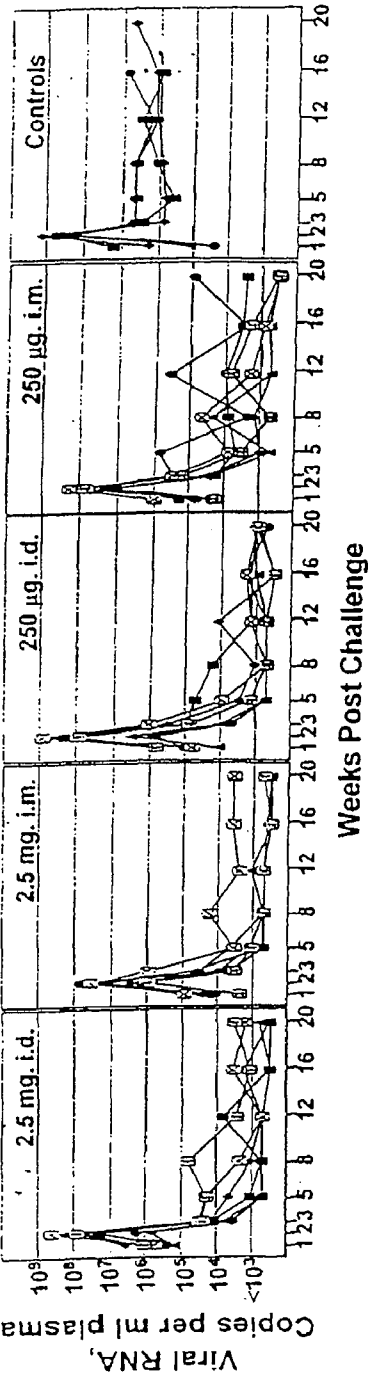


Fig. 19D

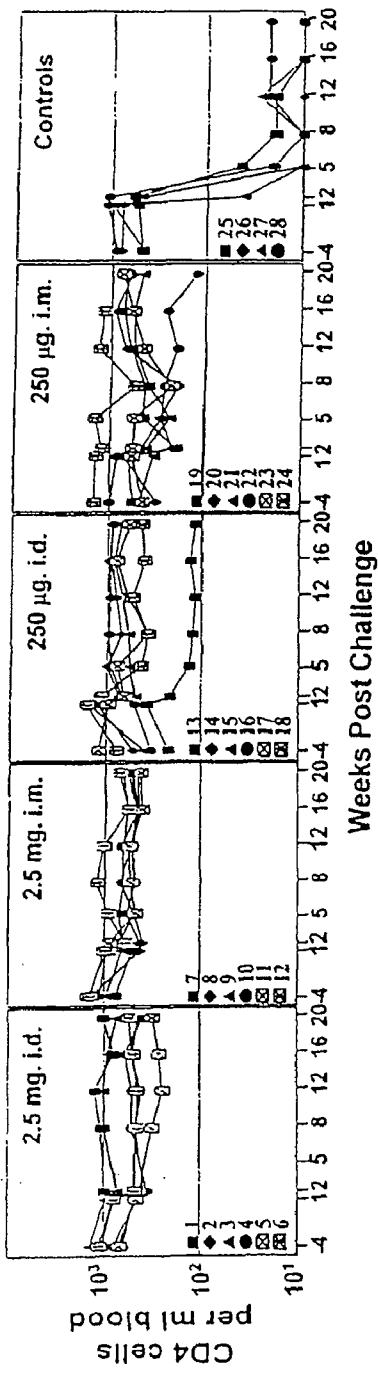


Fig. 19E

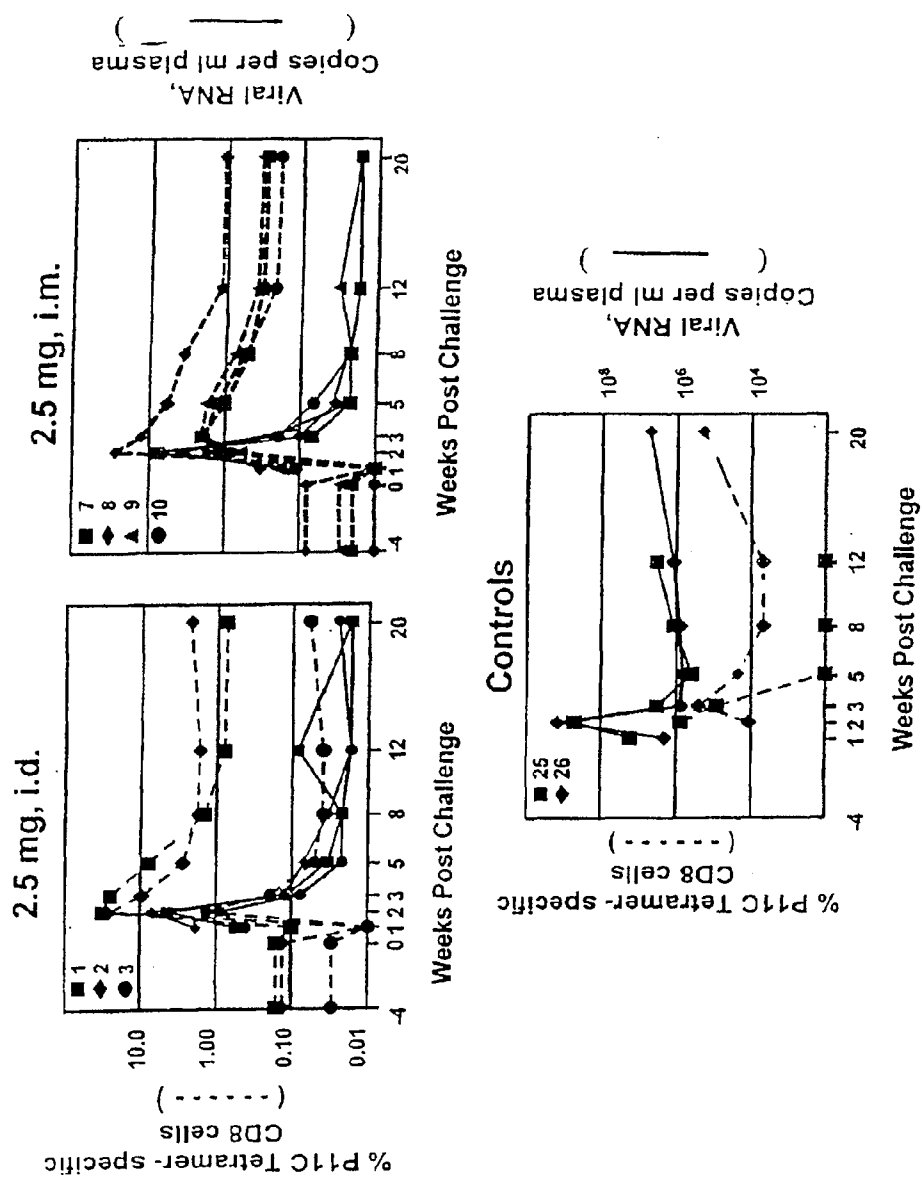


Fig. 20A

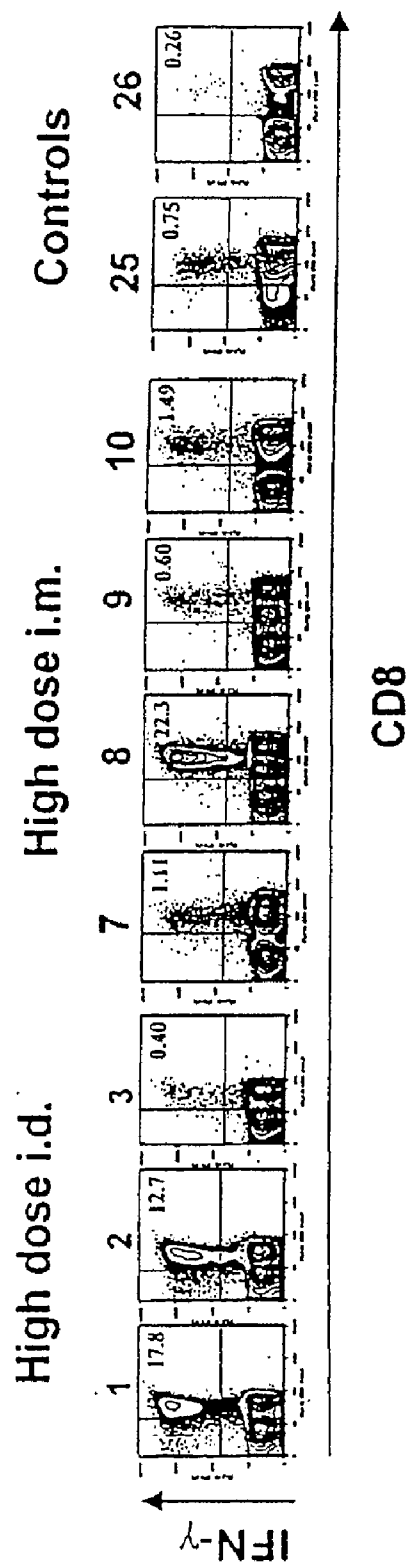


Fig. 20B

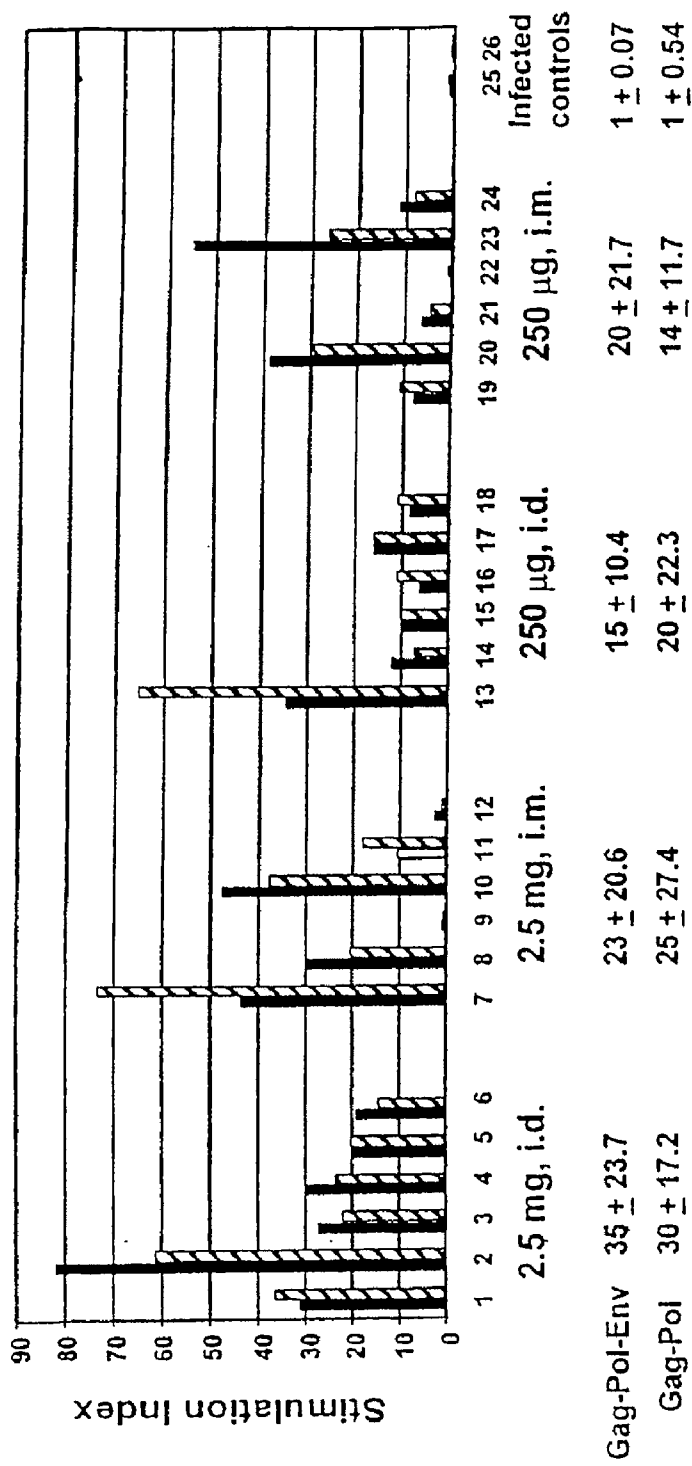
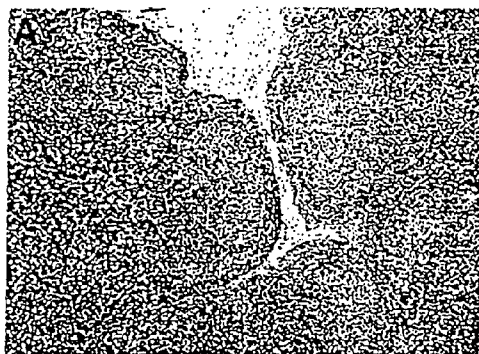
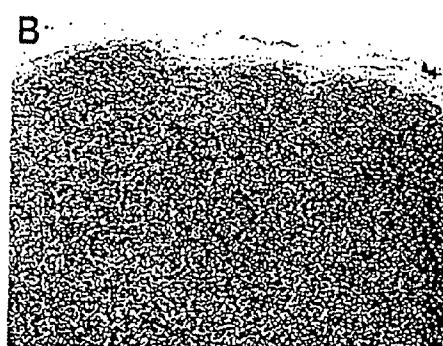


Fig. 20C



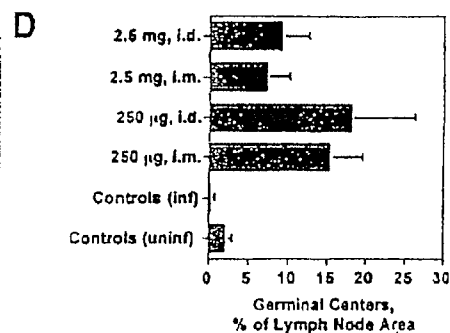
*Fig. 21A*



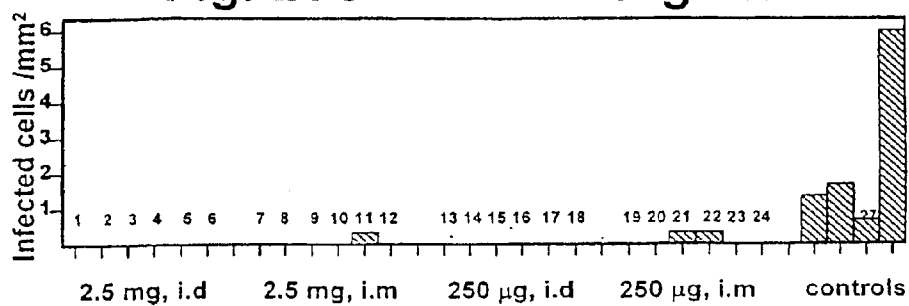
*Fig. 21B*



*Fig. 21C*



*Fig. 21D*



*Fig. 21E*



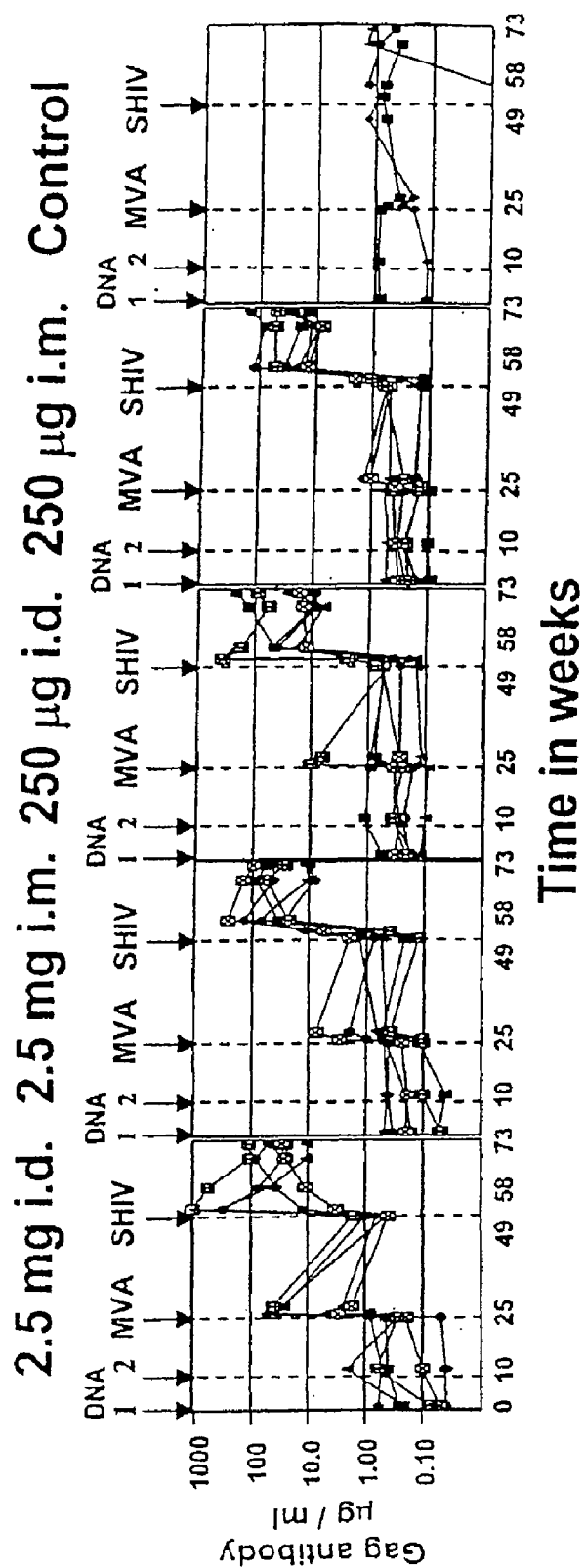


Fig. 22A

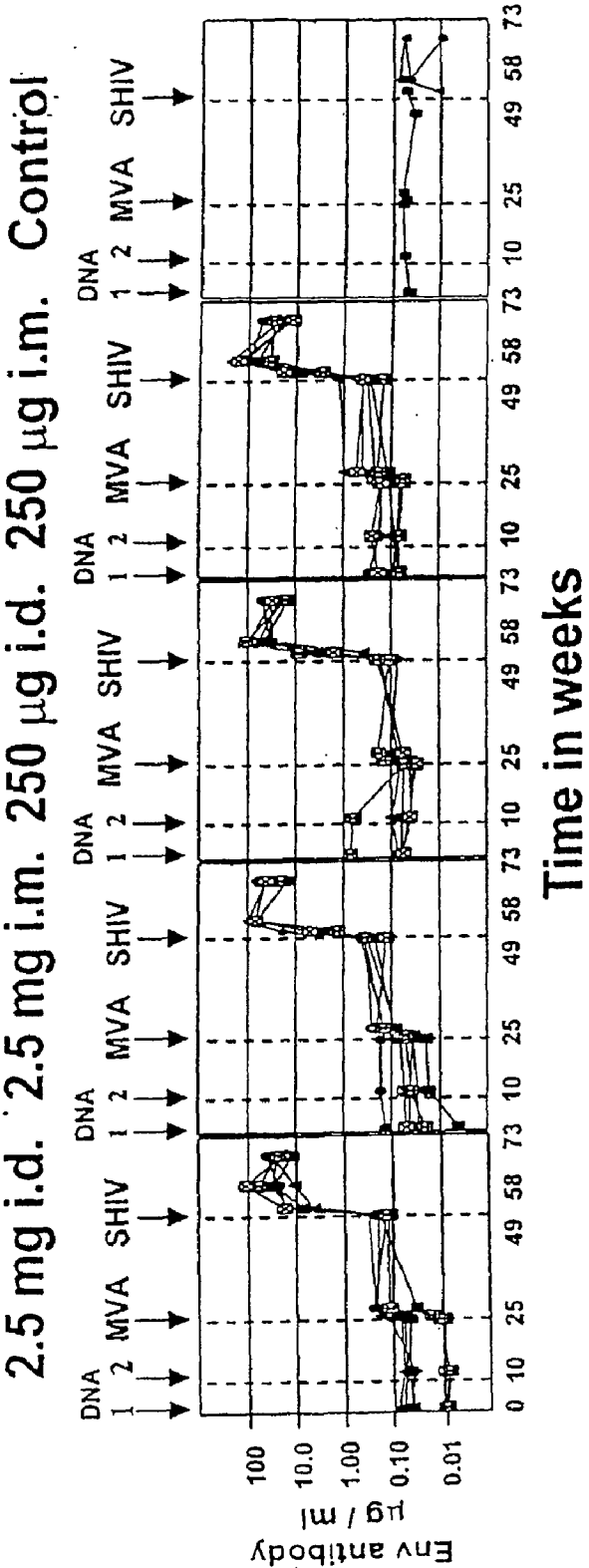
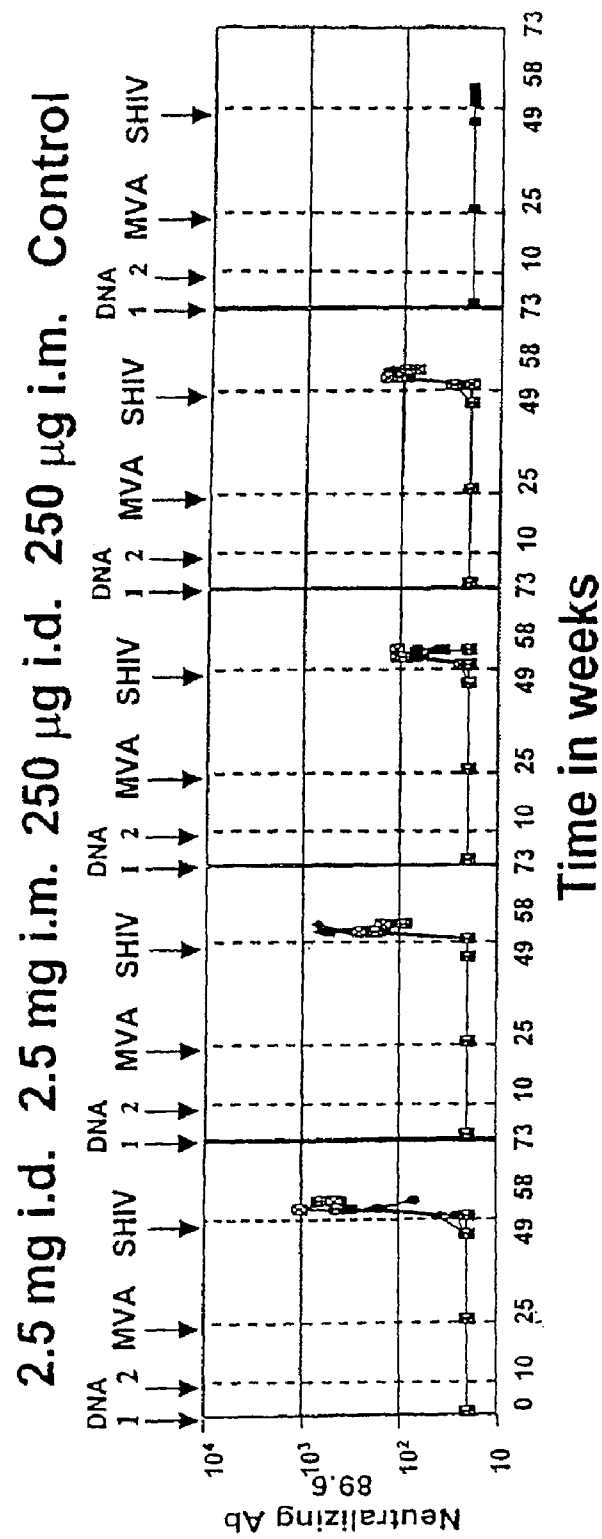
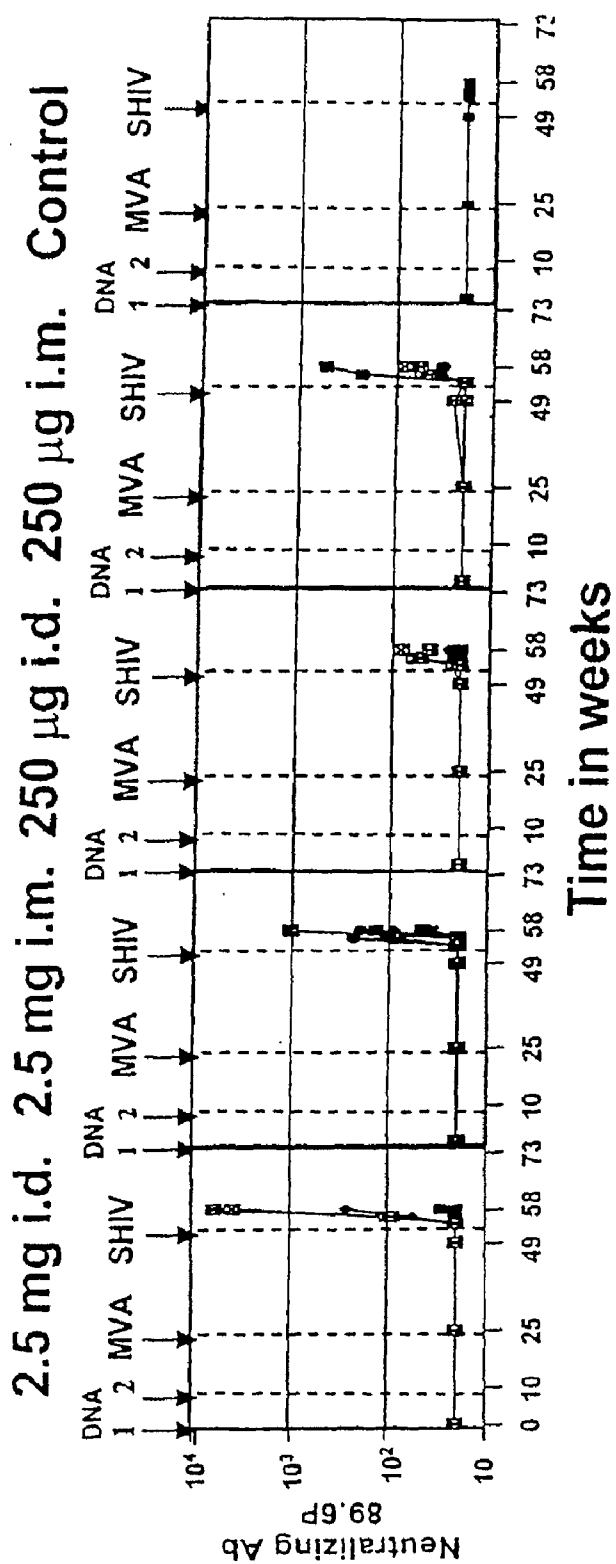


Fig. 22B



**Fig. 22C**



**Fig. 22D**

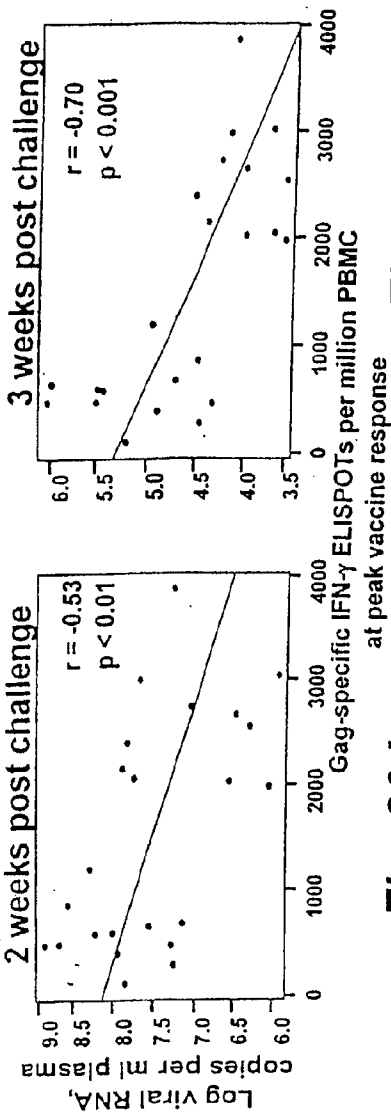


Fig. 23A

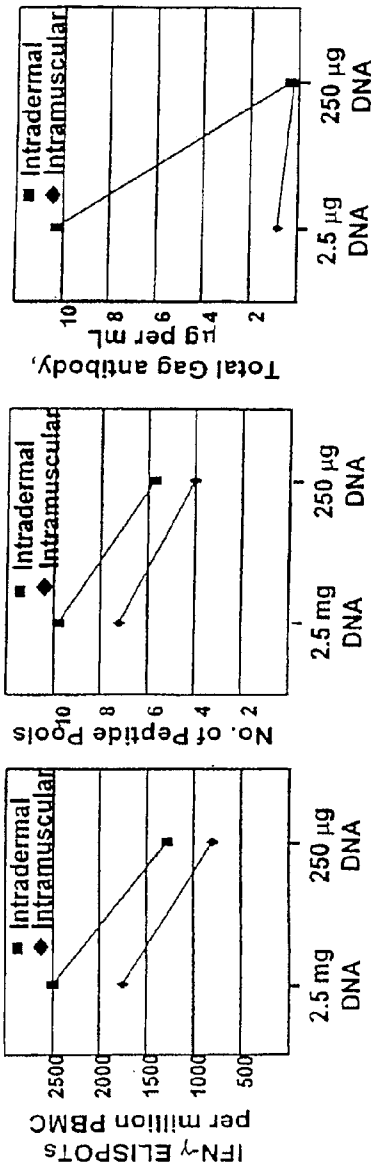


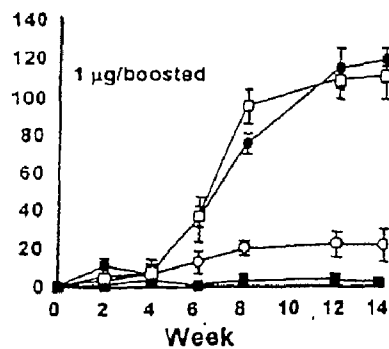
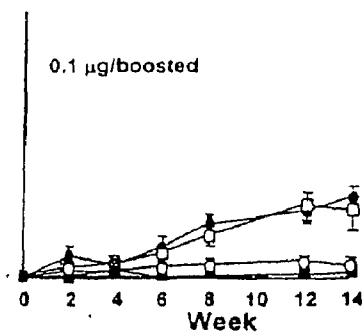
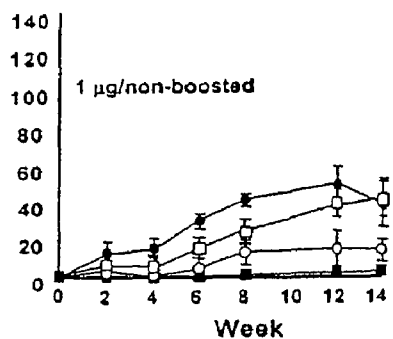
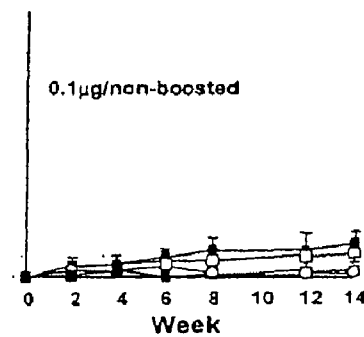
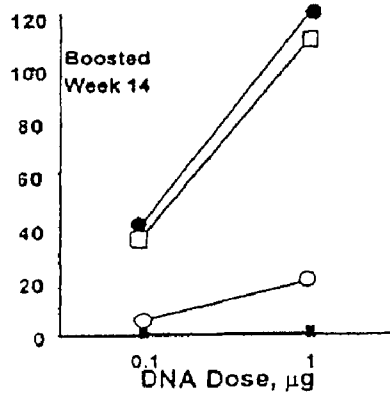
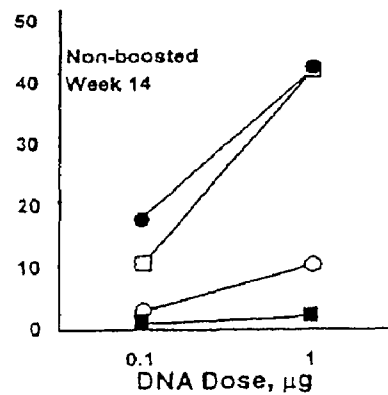
Fig. 23B

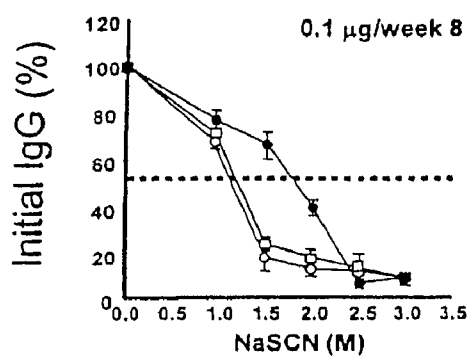
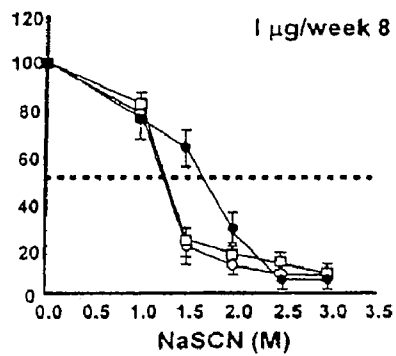
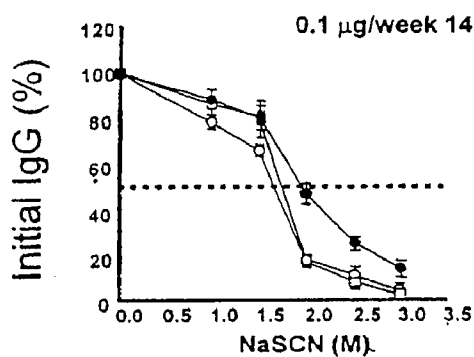
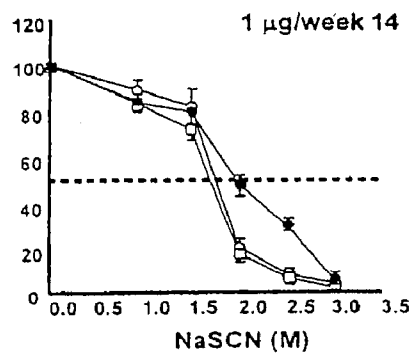
Fig. 23C

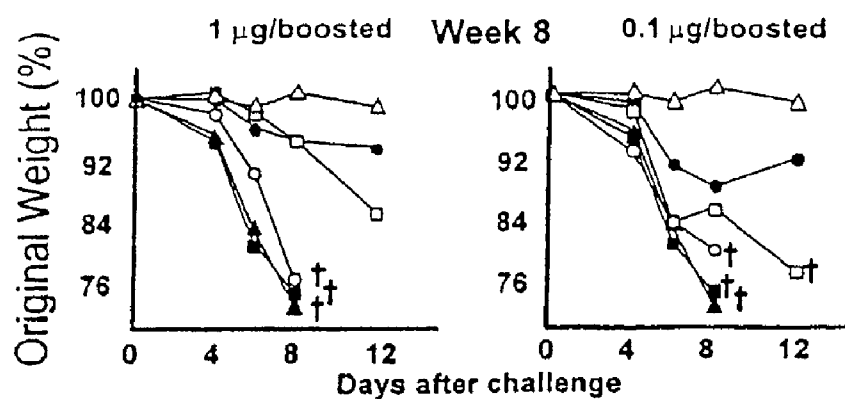
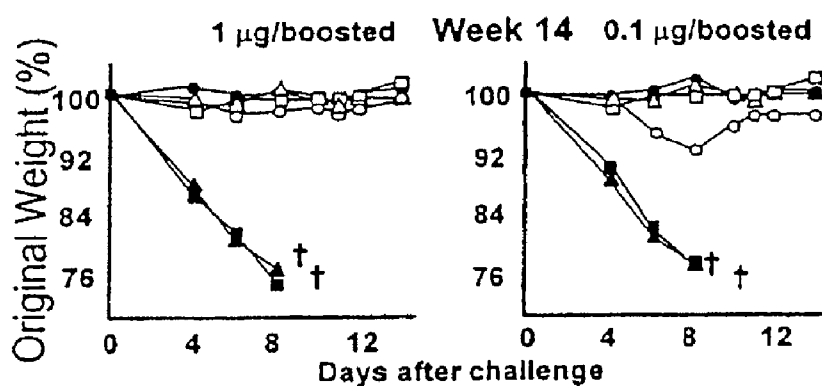
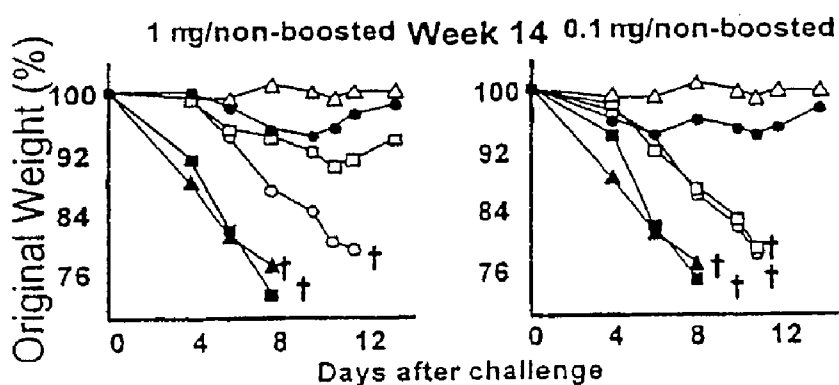
Fig. 23D

Fig. 23E

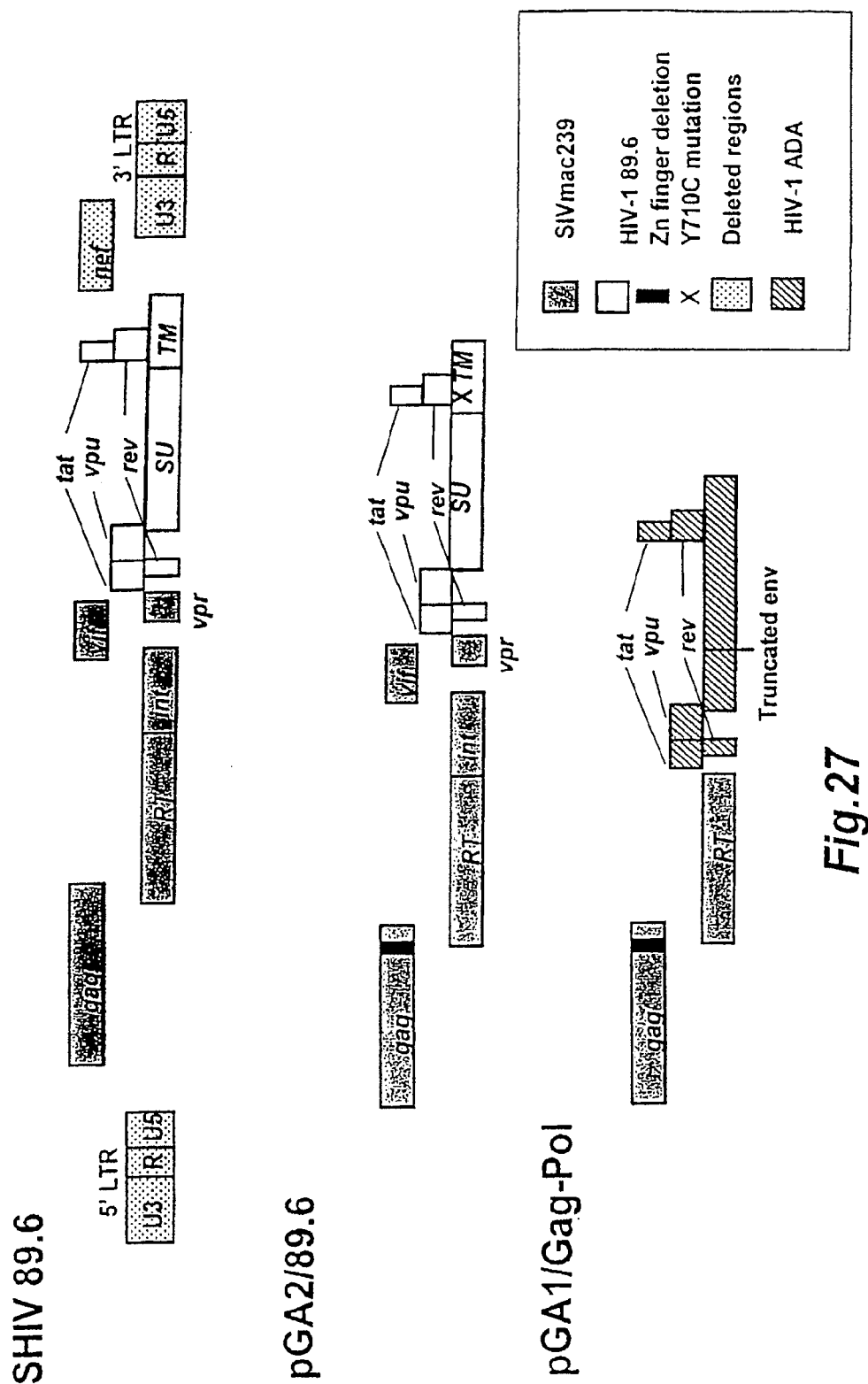
Anti-hemagglutinin IgG

**Fig. 24A****Fig. 24B****Fig. 24C****Fig. 24D****Fig. 24E****Fig. 24F**

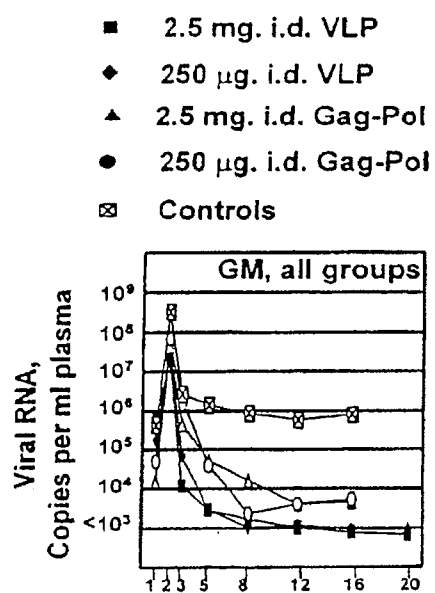
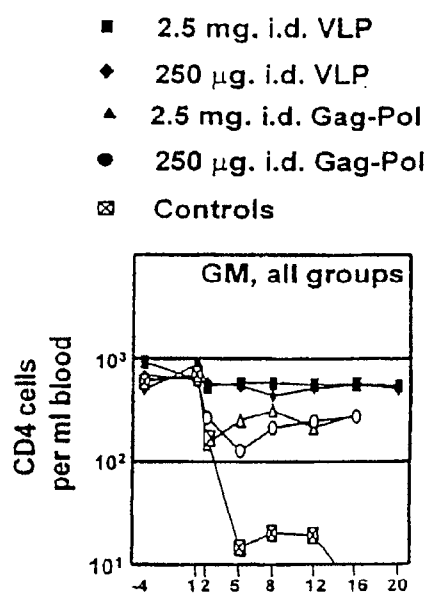
*Fig. 25A**Fig. 25B**Fig. 25C**Fig. 25D*

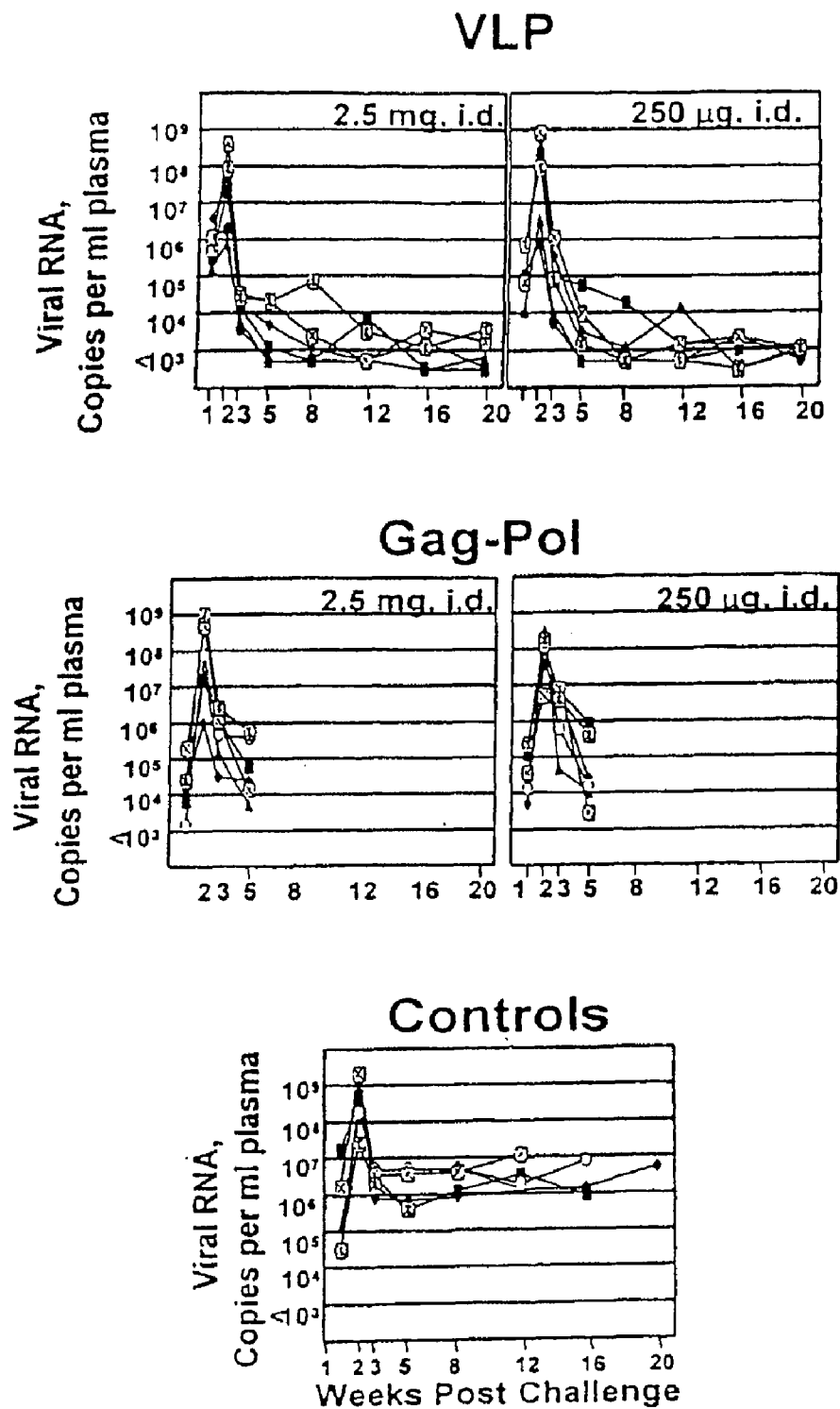
**Fig. 26A****Fig. 26B****Fig. 26C****Fig. 26D****Fig. 26E****Fig. 26F**

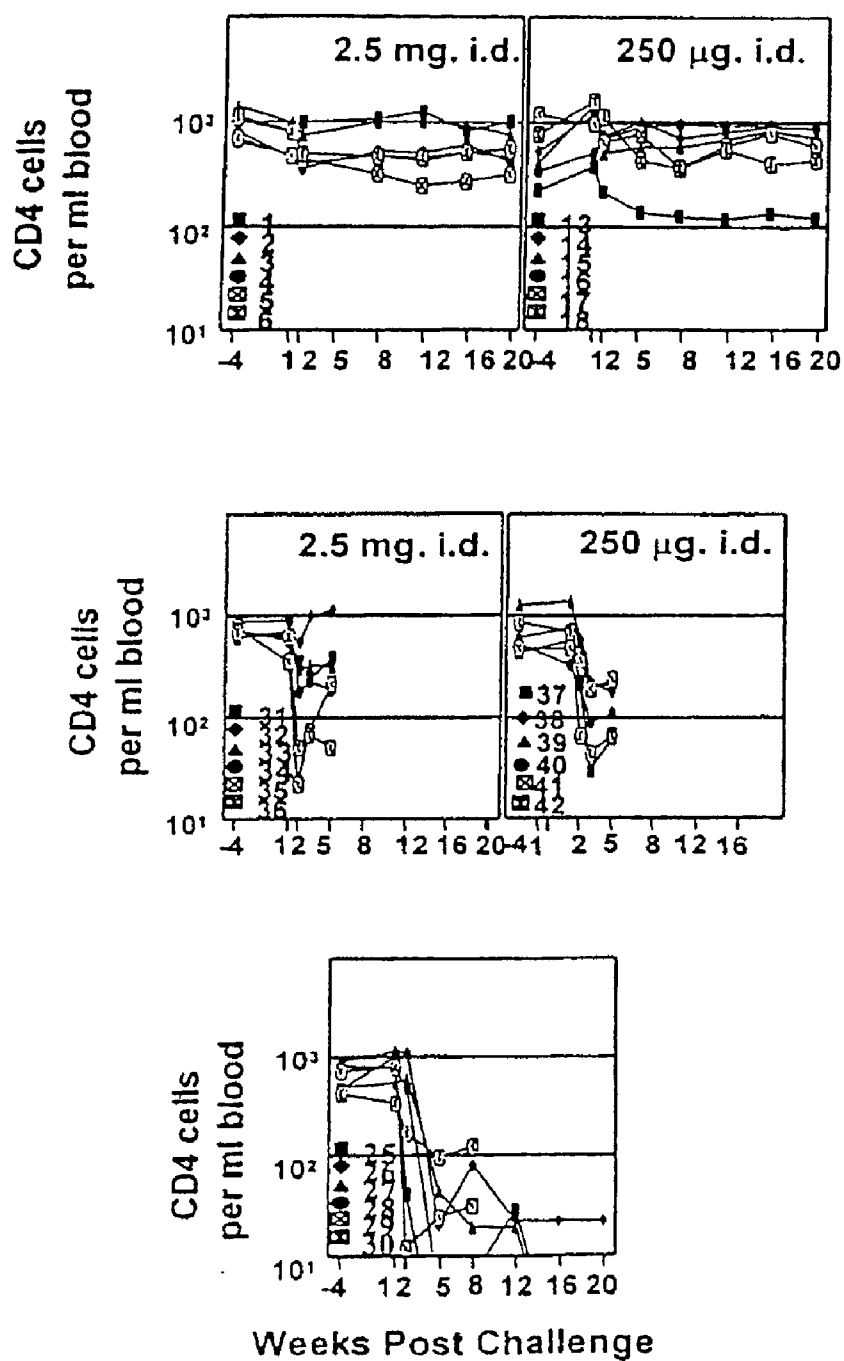


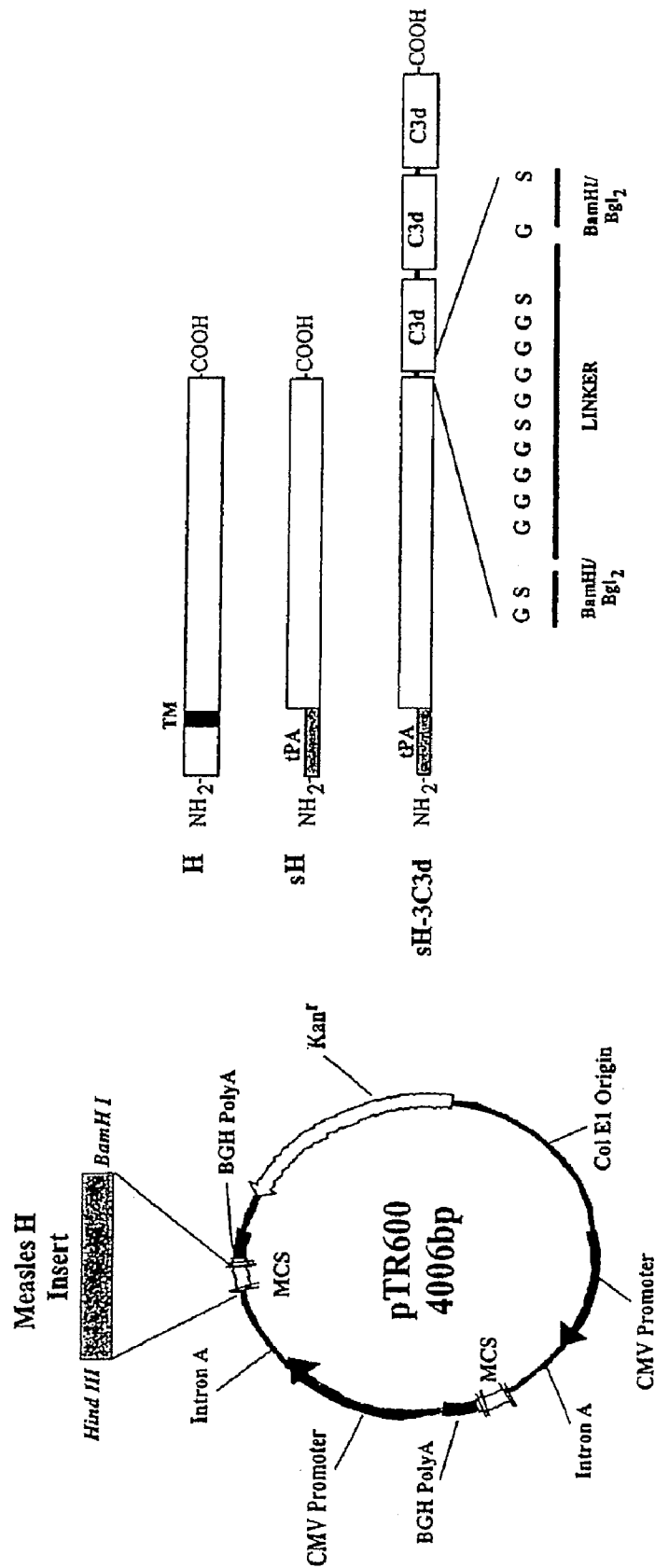


**Fig. 27**

*Fig. 28A**Fig. 28B*

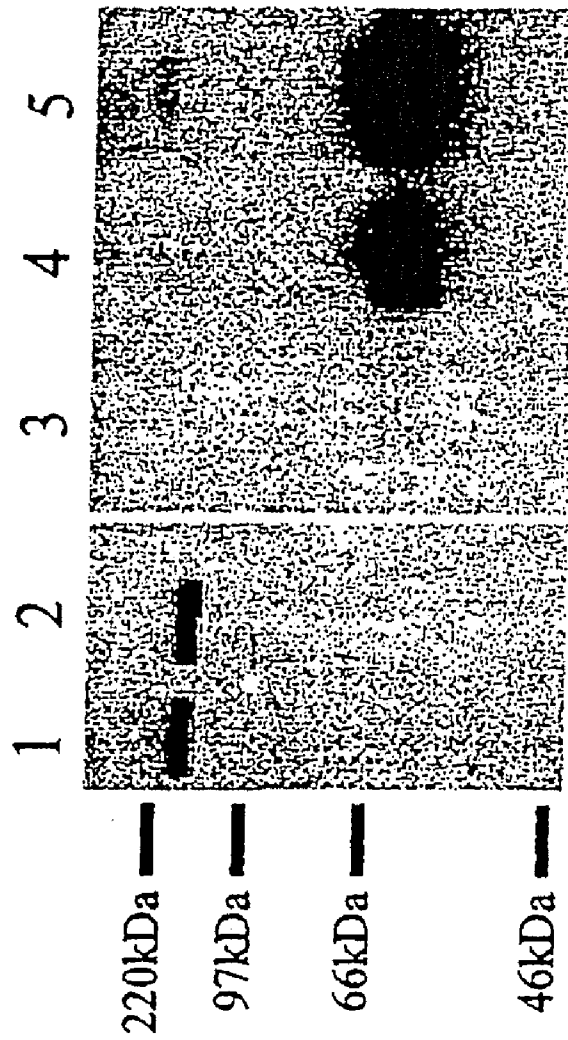
**Fig. 28C**

**Fig. 28D**



**Fig. 29B**

**Fig. 29A**



**Fig. 30**

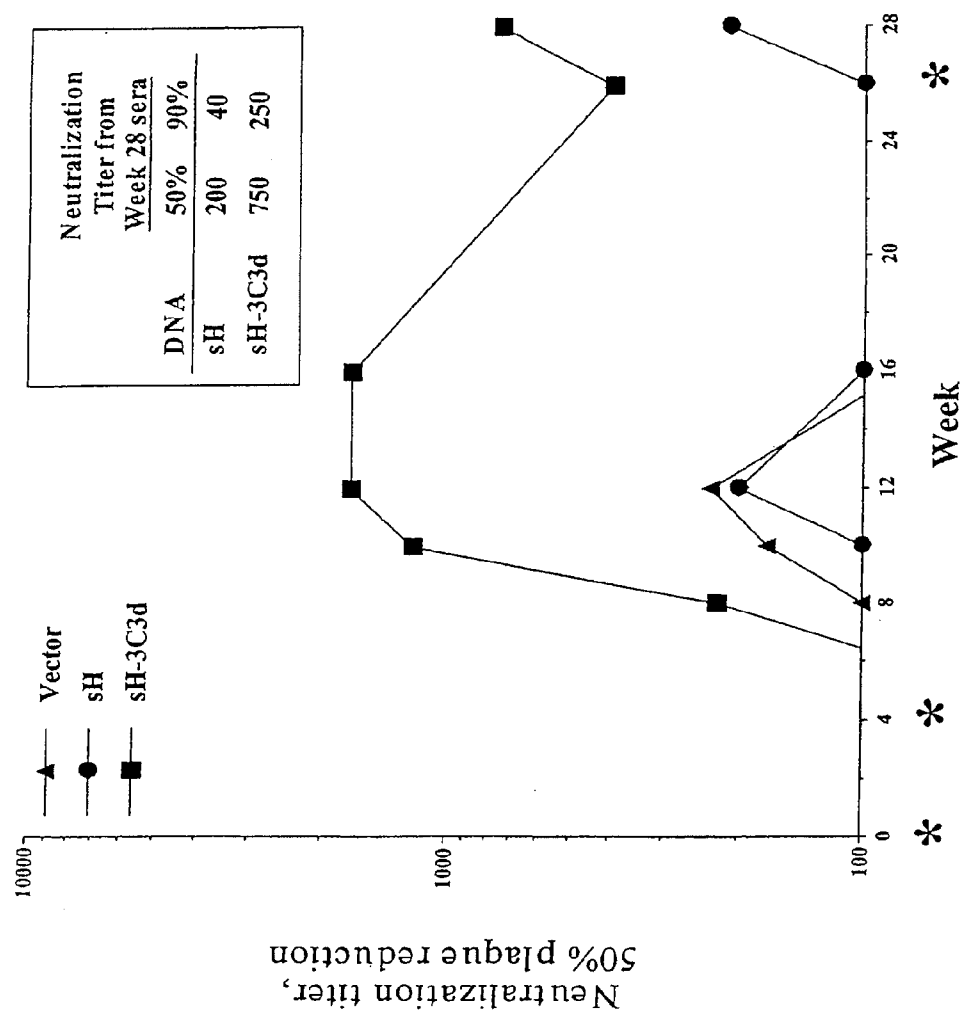


Fig. 31

## DNA EXPRESSION VECTORS AND METHODS OF USE

This application is a continuation of U.S. application Ser. No. 09/798,675, filed on Mar. 2, 2001 now abandoned, which claims the benefit of priority from U.S. provisional application Ser. No. 60/186,364, filed on Mar. 2, 2000 and Ser. No. 60/251,083, filed on Dec. 1, 2000.

### GOVERNMENT SUPPORT

Work described herein may have been supported in part by National Institutes of Health Grant 5 P01 AI43045 and National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant R21 AI44325-01. The U.S. Government may have certain rights in this invention.

### FIELD OF THE INVENTION

The present invention is directed generally to the fields of molecular genetics and immunology. More particularly, the present invention describes novel DNA expression vectors, novel vectors comprising DNA encoding an immunogenic protein, and novel methods of immunizing animals including humans by administering the novel vectors comprising DNA encoding an immunogenic protein.

### BACKGROUND OF THE INVENTION

Vaccines have had profound and long lasting effects on world health. Small pox has been eradicated, polio is near elimination, and diseases such as diphtheria, measles, mumps, pertussis, and tetanus are contained. Nonetheless, microbes remain major killers with current vaccines addressing only a handful of the infections of man and his domesticated animals. Common infectious diseases for which there are no vaccines cost the United States \$120 billion dollars per year (Robinson et al., 1997). In first world countries, emerging infections such as immunodeficiency viruses, as well as reemerging diseases like drug resistant forms of tuberculosis, pose new threats and challenges for vaccine development. The need for both new and improved vaccines is even more pronounced in third world countries where effective vaccines are often unavailable or cost-prohibitive. Recently, direct injections of antigen-expressing DNAs have been shown to initiate protective immune responses.

DNA-based vaccines use bacterial plasmids to express protein immunogens in vaccinated hosts. Recombinant DNA technology is used to clone cDNAs encoding immunogens of interest into eukaryotic expression plasmids. Vaccine plasmids are then amplified in bacteria, purified, and directly inoculated into the hosts being vaccinated. DNA typically is inoculated by a needle injection of DNA in saline, or by a gene gun device that delivers DNA-coated gold beads into skin. The plasmid DNA is taken up by host cells, the vaccine protein is expressed, processed and presented in the context of self-major histocompatibility (MHC) class I and class II molecules, and an immune response against the DNA-encoded immunogen is generated.

The historical foundations for DNA vaccines (also known as "genetic immunization") emerged concurrently from studies on gene therapy and studies using retroviral vectors. Gene therapy studies on DNA delivery into muscle revealed that pure DNA was as effective as liposome-encapsulated DNA at mediating transfection of skeletal muscle cells (Wolff et al., 1990). This unencapsulated DNA was termed "naked DNA," a fanciful term that has become popular for the description of

the pure DNA used for nucleic acid vaccinations. Gene guns, which had been developed to deliver DNA into plant cells, were also used in gene therapy studies to deliver DNA into skin. In a series of experiments testing the ability of plasmid-expressed human growth hormone to alter the growth of mice, it was realized that the plasmid inoculations, which had failed to alter growth, had elicited antibody (Tang, De Vit, and Johnston, 1992). This was the first demonstration of the raising of an immune response by an inoculated plasmid DNA. At the same time, experiments using retroviral vectors, demonstrated that protective immune responses could be raised by very few infected cells (on the order of  $10^4$ - $10^5$ ). Direct tests of the plasmid DNA that had been used to produce infectious forms of the retroviral vector for vaccination, performed in an influenza model in chickens, resulted in protective immunizations (Robinson, Hunt, and Webster, 1993).

HIV-1 is projected to infect 1% of the world's population by the year 2000, making vaccine development for this recently emergent agent a high priority for world health. Preclinical trials on DNA vaccines have demonstrated that DNA alone can protect against highly attenuated HIV-1 challenges in chimpanzees (Boyer et al., 1997), but not against more virulent SIV challenges in macaques (Lu et al., 1997). A combination of DNA priming plus an envelope glycoprotein boost has raised a neutralizing antibody-associated protection against a homologous challenge with a non-pathogenic chimera between SIV and HIV (SHIV-IIIb) (Letvin et al., 1997). More recently, a comparative trial testing eight different protocols for the ability to protect against a series of challenges with SHIV-s (chimeras between simian and human immunodeficiency viruses) revealed the best containment of challenge infections by an immunization protocol that included priming by intradermal inoculation of DNA and boosting with recombinant fowl pox virus vectors (Robinson et al., 1999). This containment of challenge infections was independent of the presence of neutralizing antibody to the challenge virus. Protocols which proved less effective at containing challenge infections included immunization by both priming and boosting by intradermal or gene gun DNA inoculations, immunization by priming with intradermal or gene gun DNA inoculations and then boosting with a protein subunit; immunization by priming with gene gun DNA inoculations and boosting with recombinant fowl pox virus, immunization with protein only, and immunization with recombinant fowl pox virus only (Robinson et al., 1999). Early clinical trials of DNA vaccines in humans have revealed no adverse effects (MacGregor et al., 1996) and the raising of cytolytic T-cells (Calarota et al., 1998). A number of studies have screened for the ability of co-transfected lymphokines and co-stimulatory molecules to increase the efficiency of immunization (Robinson and Pertmer, in press).

Disadvantages of DNA vaccine approaches include the limitation of immunizations to products encoded by DNA (e.g., proteins) and the potential for atypical processing of bacterial and parasitic proteins by eukaryotic cells. Another significant problem with existing approaches to DNA vaccines is the instability of some vaccine insert sequences during the growth and amplification of DNA vaccine plasmids in bacteria. One possible cause of instability is exposure during plasmid growth of secondary structures in vaccine inserts or the plasmid backbone that can be recognized by bacterial endonucleases.

A need exists, therefore, for DNA expression vectors that exhibit improved stability in bacterial hosts and may be safely used in animals, including humans; for eukaryotic expression of immunogenic proteins useful as vaccines against a variety of infectious diseases, including HIV-1.



## SUMMARY OF THE INVENTION

The present invention provides novel pGA constructs. The novel pGA constructs are useful as vectors for the delivery of DNA vaccines.

The present invention also provides novel pGA constructs having vaccine inserts. The pathogen vaccine inserts can include the DNA transcription unit of any virus, bacteria, parasite and/or fungi.

The present invention describes novel methods of immunizing patients by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts.

The present invention describes novel methods of immunizing patients by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts followed by booster immunizations with live vectored vaccines such as recombinant modified vaccinia Ankara (MVA) vectors comprising the same vaccine inserts.

The present invention also describes novel methods of raising multi-epitope CD8 T-cell responses by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts followed by booster immunizations with a live vectored vaccine such as recombinant modified vaccinia Ankara (MVA) vectors comprising the same vaccine inserts.

The present invention is described in more detail below.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates a novel pGA1 construct of the present invention. Designations are identities and positions of elements in the vector. Designations in italic print are unique restriction endonuclease sites useful for cloning vaccine inserts into the vector.

FIG. 2 illustrates the DNA sequence SEQ ID NO: 1 of the novel pGA1 construct shown in FIG. 1. The positions of elements in the plasmid are indicated below the nucleotide sequence.

FIG. 3 illustrates a novel pGA2 construct of the present invention. Designations are identities and positions of elements in the vector. Designations in italic print are unique restriction endonuclease sites useful for cloning vaccine inserts into the vector.

FIG. 4 illustrates the DNA sequence SEQ ID NO: 2 of the novel pGA2 construct shown in FIG. 3. The positions of elements in the plasmid are indicated below the nucleotide sequence.

FIG. 5 illustrates a novel pGA3 construct of the present invention. Designations are identities and positions of elements in the vector. Designations in italic print are unique restriction endonuclease sites useful for cloning vaccine inserts into the vector.

FIG. 6 illustrates of the DNA sequence SEQ ID NO: 3 of the novel pGA3 construct shown in FIG. 5, and the protein sequence encoded thereby (SEQ ID NO:46). The complementary strand is also shown (SEQ ID NO:39). The position of elements in the plasmid are indicated below the nucleotide sequence.

FIG. 7 compares the levels of anti-HA IgG raised by the influenza H1 hemagglutinin expressed in a pGA vector (pGA3/H1) and in the pJW4303 research vector (pJW4303/H1). BALB/c mice were immunized and boosted with a low dose (0.1 µg) or a high dose (1 µg), of the indicated plasmids using gene gun inoculations. A priming immunization was followed by a booster immunization at 4 weeks.

FIG. 8A presents a schematic of the parent wt BH10 provirus from which constructs producing non-infectious virus like particles (VLPs) were produced. Dotted regions indicate sequences that were deleted in the VLP constructs. Positions and designations of the various regions of the BH10 provirus are indicated in the rectangular boxes. The U3RU5 regions which encode the long terminal repeats contain transcriptional control elements. All other indicated regions encode proteins. For clarity, products expressed by pol (Prt, RT, Int) and env (SU and TM) are indicated.

FIG. 8B depicts the JS2 vaccine insert. This 6.7 kb vaccine insert expresses the Gag, Prt, and RT sequences of the BH10 strain of HIV-1-IIIb, Tat and Vpu proteins that are from ADA, and Rev and Env proteins that are chimeras of ADA and BH10 sequences. The Gag sequences include mutations of the zinc fingers to limit packaging of viral RNA. The RT sequences encompass three point mutations to eliminate reverse transcriptase activity. Designations are the same as in FIG. 8A. The bracketed area indicates the region of BH10 in which sequences from ADA have been substituted for the BH10 sequences to introduce a CCR-5 using Env. The x's indicate safety mutations.

FIG. 8C depicts the JS5 insert. JS5 is a 6 kb vaccine insert that expresses Gag, Prt, RT, Vpu Tat, and Rev. JS5 is comprised of the same sequences as JS2 except that sequences in Env have been deleted. The deleted sequences are indicated in FIG. 8B as a filled rectangle. Designations are the same as in FIGS. 8A and 8B. The Rev responsive element (RRE) which is in the 3' region of Env is retained in the construct.

FIGS. 9A and 9B show Gag and Env expression, respectively, for intermediates in the construction of the JS2 vaccine insert. Data are from transient transfections in 293T cells. pGA1/JS1 (ADA VLP) produced higher levels of both Gag (FIG. 9A) and Env (FIG. 9B) than wild type HIV-1 ADA or HIV-1 IIIb proviruses, and a VLP-producing DNA (dPol) used in previous studies.

FIG. 10 shows the expression of p24 capsid in transiently transfected cells by vaccine vectors expressing inserts without safety mutations (JS1 and JS4), inserts with point mutations in the zinc fingers and in RT (JS2 and JS5), and point mutations in the zinc fingers, RT, and protease (JS3 and JS6). Note that the safety mutations in the zinc fingers and RT supported active VLP expression whereas the safety mutation in Prt did not. JS2 and JS5 were chosen for continued vector development based on their high levels of expression in the presence of safety mutations.

FIGS. 11A and 11B show Gag and Env expression, respectively, of novel candidate vaccine constructs expressed by pGA vectors with and without intron A. pGA1 but not pGA2 contains intron A. pGA2/JS2 and pGA1/JS5 were chosen for use in vaccines based on their favorable levels of expression.

FIGS. 12A-12D shows Western blots of cell lysates and tissue culture supernatants from 293T cells transfected with (1) mock, (2) pGA2/JS2, and (3) pGA1/JS5, where the primary antibody was pooled from anti-HIV Ig from infected patients (FIG. 12A), anti-p24 (FIG. 12B), anti-gp120 (FIG. 12C), and anti-RT (FIG. 12D) respectively.

FIG. 13 illustrates pGA.

FIG. 14 compares Gag expression levels between pGA2/89.6, pGA1/Gag-Pol and pGA2/JS2. Comparative studies for expression were performed on transiently transfected 293T cells.

FIGS. 15A-15C show the temporal frequencies of Gag-specific T cells. FIG. 15A: Gag-specific CD8 T Cell responses raised by DNA priming and rMVA booster immunization. The schematic presents Gag-CM9-tetramer data generated in the high-dose i.d. DNA-immunized animals.

FIG. 15B: Gag-CM9-Mamu-A\*01 tetramer-specific T cells in Mamu-A\*01 vaccinated and control macaques at various times before challenge and at two weeks after challenge. The number at the upper right corner of each plot represents the frequency of tetramer-specific CD8 T cells as a % of total CD8 T cells. The numbers above each column of plots designate individual animals. FIG. 15C: Gag-specific IFN- $\gamma$  ELISPOTs in A\*01 and non-A\*01 (hatched bars) vaccinated and non-vaccinated macaques at various times before challenge and at two weeks after challenge. Three pools of approximately 10-13 Gag peptides (22-mers overlapping by 12) were used for the analyses. The numbers above data bars represent the arithmetic mean  $\pm$  the standard deviation for the ELISPOTs within each group. The numbers at the top of the graphs designate individual animals. \*, data not available; #, <20 ELISPOTs per  $1 \times 10^6$  PBMC.

FIGS. 16A-16B shows the height and breadth of IFN- $\gamma$ -producing ELISPOTs against Gag and Env in the DNA/MVA memory response. FIG. 16A: Responses against individual Gag and Env peptide pools. Data for animals within a group are designated by the same symbol. FIG. 16B: Averages of the height and breadth of ELISPOT responses for the different groups. The heights are the mean  $\pm$  the standard deviation for the sums of the Gag and Env ELISPOTs for animals in each group. The breadths are the mean  $\pm$  the standard deviation for the number of Gag and Env pools recognized by animals in each group. ELISPOT responses were determined in PBMC, during the memory phase, at 25 weeks after the rMVA booster (four weeks prior to challenge) using 7 pools of Gag peptides (approximately seven 22-mers overlapping by 12) representing about 70 amino acids of Gag sequence, and 21 pools of Env peptides (approximately ten 15-mers overlapping by 11) representing about 40 amino acids of Env sequence.

FIG. 17 shows the DNA sequence SEQ ID NO:4 of a pGA2 construct comprising the vaccine insert, where the pathogen vaccine insert. JS2 expresses Glade B HIV-1 VLP. Both the nucleotide sequence and encoded proteins (SEQ ID NOs:25-29, 40, and 41) are indicated.

FIG. 18 shows the DNA sequence of a pGA1 construct comprising the pathogen vaccine insert, where the pathogen vaccine insert. JS5 expresses clade B HIV-1 Gag-pol insert (SEQ ID NO: 5). Both the sequence and the encoded proteins (SEQ ID NOs:30-36, 42 and 43) are shown.

FIGS. 19A-19E show temporal viral loads, CD4 counts and survival after challenge of vaccinated and control animals. FIG. 19A: Geometric mean viral loads and FIG. 19B: geometric mean CD4 counts for vaccine and control groups at various weeks post-challenge. The key for the groups is in panel B. FIG. 19C: Survival curve for vaccinated and control animals. The dotted line represents all 24 vaccinated animals. FIG. 19D: viral loads and FIG. 19E: CD4 counts for individual animals in the vaccine and control groups. The key to animal numbers is presented in FIG. 19E. Assays for the first 12 weeks post challenge had a background of 1000 copies of RNA per ml of plasma. Animals with loads below 1000 were scored with a load of 500. For weeks 16 and 20, the background for detection was 300 copies of RNA/ml. Animals with levels of virus below 300 were scored at 300.

FIGS. 20A-20C show Post-challenge T-cell responses in vaccine and control groups. FIG. 20A: temporal tetramer+ cells and viral loads. FIG. 20B: Intracellular cytokine assays for IFN- $\gamma$  production in response to stimulation with the Gag-CM9 peptide at two weeks post-challenge. This ex vivo assay allows evaluation of the functional status of the peak post-challenge tetramer+ cells displayed in FIG. 15A. FIG. 20C: Proliferation assay at 12 weeks post-challenge. Gag-Pol-Env (open bars) and Gag-Pol (hatched bars) produced by

transient transfections were used for stimulation. Supernatants from mock-transfected cultures served as control antigen. Proteins were used at approximately 1  $\mu$ g per ml of p27 Gag for stimulations. Stimulation indices are the growth of cultures in the presence of viral antigens divided by the growth of cultures in the presence of mock antigen.

FIGS. 21A-21E show lymph node histomorphology and viral loads at 12 weeks post-challenge. FIG. 21A: Typical lymph node from a vaccinated macaque showing evidence of follicular hyperplasia characterized by the presence of numerous secondary follicles with expanded germinal centers and discrete dark and light zones. FIG. 21B: Typical lymph node from an infected control animal showing follicular depletion and paracortical lymphocellular atrophy. FIG. 21C: A representative lymph node from an age-matched, uninfected macaque displaying non-reactive germinal centers. FIG. 21D: The percent of the total lymph node area occupied by germinal centers was measured to give a non-specific indicator of follicular hyperplasia. Data for uninfected controls are for four age-matched rhesus macaques. FIG. 21E: Lymph node virus burden was determined by in situ hybridization using an antisense riboprobe cocktail that was complementary to SHIV-89.6 gag and pol. All of the examined nodes were inguinal lymph nodes.

FIGS. 22A-22D show temporal antibody responses following challenge. Micrograms of total Gag (FIG. 22A) or Env (FIG. 22B) antibody were determined using enzyme linked immunosorbent assays (ELISAs). The titers of neutralizing antibody for 89.6 (FIG. 22C) and 89.6P (FIG. 22D) were determined using MT-2 cell killing and neutral red staining. Titers are the reciprocal of the serum dilution giving 50% neutralization of the indicated viruses grown in human PBMC. Symbols for animals are the same as in FIG. 19.

FIGS. 23A-23E show correlations and dose response curves for the vaccine trial (FIGS. 23A and B). Inverse correlations between peak vaccine raised IFN- $\gamma$  ELISPOTs and viral loads at 2 (FIG. 23A) and 3 (FIG. 23B) weeks post-challenge. Only twenty-three of the 24 vaccinated animals are included in the correlations because of the loss of the peak DNA/MVA ELISPOT sample for animal 3 (see FIG. 15C). (FIG. 23C) Dose response curves for the average height of Gag ELISPOTS at the peak DNA-MVA response (data from FIG. 15C). (FIG. 23D) Dose response curve for the breadth of the DNA/MVA memory ELISPOT response (data from FIG. 16B). (FIG. 23E) Dose response curves for the peak anti-Gag antibody response post the MVA booster (data from FIG. 22A). The different doses of DNA raised different levels of ELISPOT and antibody responses ( $P < 0.05$ ). The route of DNA inoculation had a significant effect on the antibody ( $P = 0.02$ ), but not the ELISPOT response.

FIG. 24 shows anti-HA IgG raised by gene gun inoculation of DNAs expressing HA proteins.

FIG. 25. Shows avidity of the anti HA IgG raised by the three different HA DNA vaccines.

FIG. 26 shows protection from weight loss after virus challenge.

FIG. 27 illustrates the importance of including Env in the vaccine.

FIGS. 28A-28D illustrates the importance of including Env in vaccines administered to animals challenged interectally with SHIV-89.6P.

FIG. 29 is a schematic representation of vector DNA vaccine constructs.

FIG. 30 shows Western blot results showing expression of vaccine constructs in vitro.

FIG. 31 is a temporal curve of measles virus neutralizing antibody.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention relates to novel vectors, novel vectors comprising pathogen vaccine inserts, and novel methods of immunizing patients against a pathogen. The novel immunization methods elicit both cell-mediated and humoral immune responses that may limit the infection, spread or growth of the pathogen and result in protection against subsequent challenge by the pathogen.

Classic references for DNA vaccines include the first demonstration of the raising of an immune response (Tang, De Vit, and Johnston, 1992); the first demonstration of cytotoxic T-cell (Tc)-mediated immunity (Ulmer et al., 1993); the first demonstration of the protective efficacy of intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), intranasal (i.n.), and gene gun (g.g.) immunizations (Fynan et al., 1993; Robinson, Hunt, and Webster, 1993); the first use of genetic adjuvants (Xiang and Ertl, 1995); the first use of library immunizations (Barry, Lai, and Johnston, 1995); and the first demonstration of the ability to modulate the T-helper type of an immune response by the method of DNA delivery (Feltquate et al., 1997). A highly useful web site compiling DNA vaccine information can be found at <http://www.genweb.com/Dnavax/dnavax.html>.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

#### DEFINITIONS

The term "nucleic acid" as used herein refers to any natural and synthetic linear and sequential arrays of nucleotides and nucleosides, for example cDNA, genomic DNA, mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. For ease of discussion, such nucleic acids may be collectively referred to herein as "constructs," "plasmids," or "vectors." Representative examples of the nucleic acids of the present invention include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, but not limited to, pBR322, animal viral vectors such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retrovirus, and the like, vectors derived from bacteriophage nucleic acid, and synthetic oligonucleotides like chemically synthesized DNA or RNA. The term "nucleic acid" further includes modified or derivatised nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatised nucleotides such as biotin-labeled nucleotides.

The term "isolated nucleic acid" as used herein refers to a nucleic acid with a structure (a) not identical to that of any naturally occurring nucleic acid or (b) not, identical to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes, and includes DNA, RNA, or derivatives or variants thereof. The term includes, but is not limited to, the following: (a) a DNA which has the sequence of part of a naturally occurring genomic molecule but is not flanked by at least one of the coding sequences that flank that part of the molecule in the genome of the species in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic nucleic acid of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any vector or naturally occurring genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), ligase chain reaction (LCR) or chemical synthesis, or a

restriction fragment; (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein, and (e) a recombinant nucleotide sequence that is part of a hybrid sequence that is not naturally occurring.

It is advantageous for some purposes that a nucleotide sequence is in purified form. The term "purified" in reference to nucleic acid represents that the sequence has increased purity relative to the natural environment.

As used herein the terms "polypeptide" and "protein" refer to a polymer of amino acids of three or more amino acids in a serial array, linked through peptide bonds. The term "polypeptide" includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term "polypeptides" contemplates polypeptides as defined above that are encoded by nucleic acids, produced through recombinant technology, isolated from an appropriate source, or are synthesized. The term "polypeptides" further contemplates polypeptides as defined above that include chemically modified amino acids or amino acids covalently or noncovalently linked to labeling ligands.

The term "fragment" as used herein to refer to a nucleic acid (e.g., cDNA) refers to an isolated portion of the subject nucleic acid constructed artificially (e.g., by chemical synthesis) or by cleaving a natural product into multiple pieces, using restriction endonucleases or mechanical shearing, or a portion of a nucleic acid synthesized by PCR, DNA polymerase or any other polymerizing technique well known in the art, or expressed in a host cell by recombinant nucleic acid technology well known to one of skill in the art. The term "fragment" as used herein may also refer to an isolated portion of a polypeptide, wherein the portion of the polypeptide is cleaved from a naturally occurring polypeptide by proteolytic cleavage by at least one protease, or is a portion of the naturally occurring polypeptide synthesized by chemical methods well known to one of skill in the art.

The term "gene" or "genes" as used herein refers to nucleic acid sequences (including both RNA or DNA) that encode genetic information for the synthesis of a whole RNA, a whole protein, or any portion of such whole RNA or whole protein. Genes that are not naturally part of a particular organism's genome are referred to as "foreign genes", "heterologous genes" or "exogenous genes" and genes that are naturally a part of a particular organism's genome are referred to as "endogenous genes".

The term "expressed" or "expression" as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. The term "expressed" or "expression" as used herein also refers to the translation from said RNA nucleic acid molecule to give a protein or polypeptide or a portion thereof.

As used herein, the term "locus" or "loci" refers to the site of a gene on a chromosome. Pairs of genes control hereditary traits, each in the same position on a pair of chromosomes. These gene pairs, or alleles, may both be dominant or both be recessive in expression of that trait. In either case, the individual is said to be homozygous for the trait controlled by that gene pair. If the gene pair (alleles) consists of one dominant and one recessive trait, the individual is heterozygous for the trait controlled by the gene pair. Natural variation in genes or nucleic acid molecules caused by, for example, recombination events or resulting from mutation, gives rise to allelic variants with similar, but not identical, nucleotide sequences. Such allelic variants typically encode proteins with similar activity to that of the protein encoded by the gene to which they are compared, because natural selection typically selects against variations that alter function. Allelic variants can also

comprise alterations in the untranslated regions of the gene as, for example, in the 3' or 5' untranslated regions or can involve alternate splicing of a nascent transcript, resulting in alternative exons being positioned adjacently.

The term "transcription regulatory sequences" as used herein refers to nucleotide sequences that are associated with a gene nucleic acid sequence and which regulate the transcriptional expression of the gene. The "transcription regulatory sequences" may be isolated and incorporated into a vector nucleic acid to enable regulated transcription in appropriate cells of portions of the vector DNA. The "transcription regulatory sequence" may precede, but are not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that may be transcribed into mRNA. Transcriptional regulatory sequences may also be located within a protein coding region, in regions of a gene that are identified as "intron" regions, or may be in regions of nucleic acid sequence that are in the region of nucleic acid.

The term "coding region" as used herein refers to a continuous linear arrangement of nucleotides that may be translated into a protein. A full length coding region is translated into a full length protein; that is, a complete protein as would be translated in its natural state absent any post-translational modifications. A full length coding region may also include any leader protein sequence or any other region of the protein that may be excised naturally from the translated protein.

The term "probe" as used herein, when referring to a nucleic acid, refers to a nucleotide sequence that can be used to hybridize with and thereby identify the presence of a complementary sequence, or a complementary sequence differing from the probe sequence but not to a degree that prevents hybridization under the hybridization stringency conditions used. The probe may be modified with labels such as, but not only, radioactive groups, biotin, or any other label that is well known in the art.

The term "nucleic acid vector" as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded plasmid can be linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the plasmid vector. A nucleic acid can be inserted into a vector by cutting the vector with restriction enzymes and ligating the pieces together. The nucleic acid molecule can be RNA or DNA.

The term "expression vector" as used herein refers to a nucleic acid vector that may further include at least one regulatory sequence operably linked to a nucleotide sequence coding for the Mago protein. Regulatory sequences are well recognized in the art and may be selected to ensure good expression of the linked nucleotide sequence without undue experimentation by those skilled in the art. As used herein, the term "regulatory sequences" includes promoters, enhancers, and other elements that may control expression. Standard molecular biology textbooks such as *Sambrook et al. eds "Molecular Cloning: A Laboratory Manual"* 2nd ed. Cold Spring Harbor Press (1989) may be consulted to design suitable expression vectors, promoters, and other expression control elements. It should be recognized, however, that the choice of a suitable expression vector depends upon multiple factors including the choice of the host cell to be transformed and/or the type of protein to be expressed.

The terms "transformation" and "transfection" as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid

into a prokaryotic or eukaryotic organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt such as, but not only a calcium or magnesium salt, an electric field, detergent, or liposome mediated transfection, to render the host cell competent for the uptake of the nucleic acid molecules.

The term "recombinant cell" refers to a cell that has a new combination of nucleic acid segments that are not covalently linked to each other in nature. A new combination of nucleic acid segments can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. A recombinant cell can be a single eukaryotic cell, or a single prokaryotic cell, or a mammalian cell. The recombinant cell can harbor a vector that is extragenomic. An extragenomic nucleic acid vector does not insert into the cell's genome. A recombinant cell can further harbor a vector or a portion thereof that is intragenomic. The term intragenomic defines a nucleic acid construct incorporated within the recombinant cell's genome.

The term "recombinant nucleic acid" as used herein refers to combinations of at least two nucleic acid sequences that are not naturally found in a eukaryotic or prokaryotic cell. The nucleic acid sequences may include, but are not limited to nucleic acid vectors, gene expression regulatory elements, origins of replication, sequences that when expressed confer antibiotic resistance, and protein-encoding sequences. The term "recombinant polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

The term "patients," as used herein, refers to animals, preferably mammals, and more preferably humans.

The term "immunizing" or "immunization," as used herein, refers to the production of an immune response in a patient that protects (partially or totally) from the manifestations of infection (i.e., disease) caused by a pathogen. A patient immunized by the present invention will not be infected by the pathogen or will be infected to a lesser extent than would occur without immunization. Immunizations may be either prophylactic or therapeutic in nature. That is, both previously uninfected and infected patients may be immunized with the present invention.

The term "DNA transcription unit" as used herein "refers to a polynucleotide sequence that includes at least two components: antigen-encoding DNA and transcriptional promoter elements. A DNA transcription unit may optionally include additional sequences, such as enhancer elements, splicing signals, termination and polyadenylation signals, viral replicons, and/or bacterial plasmid sequences. The DNA transcription unit can be produced by a number of known methods. For example, DNA encoding the desired antigen can be inserted into an expression vector to construct the DNA transcription unit, as described in Maniatis et al, *Molecular Cloning: A Laboratory Manual*, 2d, Cold Spring Harbor Laboratory Press (1989), the disclosure of which is incorporated by reference in its entirety.

The term "vaccine insert" as used herein refers to the DNA transcription unit of a pathogen. Preferably, the vaccine insert is a DNA transcription unit that can generate an immune responses in a patient. For example, the vaccine insert is a pathogen vaccine insert encoding antigens derived from any virus, bacteria, parasite and/or fungi. Exemplary viruses include herpesvirus, orthomyxoviruses, rhinoviruses, picornaviruses, adenoviruses, paramyxoviruses, coronaviruses, rhabdoviruses, togaviruses, flaviviruses, bunyaviruses,

rubella virus, reovirus, measles, hepadna viruses, Ebola, retroviruses (including human immunodeficiency virus), and the like. Exemplary bacteria include tuberculosis, mycobacteria, spirochetes, rickettsias, chlamydia, mycoplasma and the like. Exemplary parasites include malaria and the like. Exemplary fungi include yeasts, molds, and the like. One skilled in the art will appreciate that this list does not include all potential pathogens against which a protective immune response can be generated by the methods described herein.

The term "antigen" as used herein refers to any protein, carbohydrate, or other moiety expressed by a pathogen that is capable of eliciting a protective response against a pathogen. The antigen may or may not be a structural component of the pathogen. Also contemplated to be within the term "antigen" are encoded antigens that can be translation products or polypeptides of various lengths. Antigens undergo normal host cell modifications such as glycosylation, myristoylation or phosphorylation. In addition, they can be designed to undergo intracellular, extracellular or cell-surface expression. Furthermore, they can be designed to undergo assembly and release from cells.

As used herein, the term "adjuvant" means a substance added to a vaccine to increase a vaccine's immunogenicity. The mechanism of how an adjuvant operates is not entirely known. Some adjuvants are believed to enhance the immune response by slowly releasing the antigen, while other adjuvants are strongly immunogenic in their own right and are believed to function synergistically. Known vaccine adjuvants include, but are not limited to, oil and water emulsions (for example, complete Freund's adjuvant and incomplete Freund's adjuvant), *Corynebacterium parvum*, Bacillus Calmette Guérin, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, "REGRESSIN" (Vetrepharm, Athens, Ga.), "AVRIDINE" (N,N-diocetadecyl-N',N'-bis(2-hydroxyethyl)-propanediamine), paraffin oil, and muramyl dipeptide. Adjuvants also encompass genetic adjuvants such as immunomodulatory molecules encoded in a co-inoculated DNA. The co-inoculated DNA can be in the same vaccine construct as the vaccine immunogen or in a separate-DNA vector.

As used herein, the term "pharmaceutically acceptable carrier" means a vehicle for containing the vaccine that can be injected into a bovine host without adverse effects. Suitable pharmaceutically acceptable carriers known in the art include, but are not limited to, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

The terms "selectable marker gene" as used herein refer to an expressed gene that allows for the selection of a population of cells containing the selectable marker gene from a population of cells not having the expressed selectable marker gene. For example, the "selectable marker gene" may be an "antibiotic resistance gene" that can confer tolerance to a specific antibiotic by a microorganism that was previously adversely affected by the drug. Such resistance may result from a mutation or the acquisition of resistance due to plasmids containing the resistance gene transforming the microorganism.

The term "terminator sequence" or "terminator" as used herein refers to nucleotide sequences that function to stop transcription. The terms "transcription" or "transcribe" as used herein refers to the process by which RNA molecules are

formed upon DNA templates by complementary base pairing. This process is mediated by RNA polymerase.

The term "VLP" as used herein refers to virus-like particles and, as used, also refers to aggregates of viral proteins.

The major immunological advantage of DNA-based immunizations is the ability of the immunogen to be presented by both MHC class I and class II molecules. Endogenously synthesized proteins readily enter processing pathways for the loading of peptide epitopes onto MHC I as well as MHC II molecules. MHC I-presented epitopes raise cytotoxic T-cells (Tc) responses whereas MHC II-presented epitopes raise helper T-cells (Th). By contrast, immunogens that are not synthesized in cells are largely restricted to the loading of MHC II epitopes and the raising of Th but not Tc. When compared with live attenuated vaccines or recombinant viral vectors that produce immunogens in cells and raise both Th and Tc, DNA vaccines have the advantages of not being infectious and of focusing the immune response on only those antigens desired for immunization. DNA vaccines also are advantageous because they can be manipulated relatively easily to raise type 1 or type 2 T-cell help. This allows a vaccine to be tailored for the type of immune response that will be mobilized to combat an infection. DNA vaccines are also cost effective because of the ease with which plasmids can be constructed using recombinant DNA technology, the ability to use a generic method for vaccine production (growth and purification of plasmid DNA), and the stability of DNA over a wide range of temperatures.

The best immune responses are achieved using highly active expression vectors modeled on those developed for the production of recombinant proteins (Robinson and Pertmer, 1998). The most frequently used transcriptional control elements include a strong promoter. One such promoter suitable for use is the cytomegalovirus (CMV) intermediate early promoter, although other promoters may be used in a DNA vaccine without departing from the scope the present invention. Other transcriptional control elements useful in the present invention include a strong polyadenylation signal such as, for example, that derived from a bovine growth hormone encoding gene, or a rabbit  $\beta$  globin polyadenylation signal (Bohm et al., 1996; Chapman et al., 1991; Hartikka et al., 1996; Manthorpe et al., 1993; Montgomery et al., 1993). The CMV immediate early promoter may be used with or without intron A (Chapman et al., 1991). The presence of intron A increases the expression of many antigens from RNA viruses, bacteria, and parasites, presumably by providing the expressed RNA with sequences which support processing and function as an eukaryotic mRNA. It will be appreciated that expression also may be enhanced by other methods known in the art including, but not limited to, optimizing the codon usage of prokaryotic mRNAs for eukaryotic cells (Andre et al., 1998; Uchijima et al., 1998). Multi-cistronic vectors may be used to express more than one immunogen or an immunogen and an immunostimulatory protein (Iwasaki et al., 1997a; Wild et al., 1998).

Immunogens can also be engineered to be more or less effective for raising antibody or Tc by targeting the expressed antigen to specific cellular compartments. For example, antibody responses are raised more effectively by antigens that are displayed on the plasma membrane of cells, or secreted therefrom, than by antigens that are localized to the interior of cells (Boyle, Koniaras, and Lew, 1997; Inchauspe et al., 1997). Tc responses may be enhanced by using N-terminal ubiquitination signals which target the DNA-encoded protein to the proteasome causing rapid cytoplasmic degradation and more efficient peptide loading into the MHC I pathway (Rodriguez, Zhang, and Whitton, 1997; Tobery and Siliciano,

1997; Wu and Kipps, 1997). For a review on the mechanistic basis for DNA-raised immune responses, refer to Robinson and Pertmer, *Advances in Virus Research*, vol. 53, Academic Press (2000), the disclosure of which is incorporated herein by reference in its entirety.

The effects of different conformational forms of proteins on antibody responses, the ability of strings of MHC I epitopes (minigenes) to raise Tc responses, and the effect of fusing an antigen with immune-targeting proteins have been evaluated using defined inserts. Ordered structures such as virus-like particles appear to be more effective than unordered structures at raising antibody (Fomsgaard et al., 1998). This is likely to reflect the regular array of an immunogen being more effective than a monomer of an antigen at cross-linking Ig-receptors and signaling a B-cell to multiply and produce antibody. Recombinant DNA molecules encoding a string of MHC epitopes from different pathogens can elicit Tc responses to a number of pathogens (Hanke et al., 1998b). These strings of Tc epitopes are most effective if they also include a Th epitope (Maecker et al., 1998; Thomson et al., 1998).

Another approach to manipulating immune responses is to fuse immunogens to immunotargeting or immunostimulatory molecules. To date, the most successful of these fusions have targeted secreted immunogens to antigen presenting cells (APC) or lymph nodes (Boyle, Brady, and Lew, 1998). Fusion of a secreted form of human IgG with CTLA-4 increased antibody responses to the IgG greater than 1000-fold and changed the bias of the response from complement (C') dependent to C'-independent antibodies.

Fusions of human IgG with L-selectin also increased antibody responses but did not change the C'-binding characteristics of the raised antibody. The immunogen fused with L-selectin was presumably delivered to lymph nodes by binding to the high endothelial venules, which serve as portals. Fusions between antigens and cytokine cDNAs have resulted in more moderate increases in antibody, Th, and Tc responses (Hakim, Levy, and Levy, 1996; Maecker et al., 1997). IL-4-fusions have increased antibody responses, whereas IL-12 and IL-1 $\beta$  have enhanced T-cell responses.

Two approaches to DNA delivery are injection of DNA in saline using a hypodermic needle or gene gun delivery of DNA-coated gold beads. Saline injections deliver DNA into extracellular spaces, whereas gene gun deliveries bombard DNA directly into cells. The saline injections require much larger amounts of DNA (100-1000 times more) than the gene gun (Fynan et al., 1993). These two types of delivery also differ in that saline injections bias responses towards type 1 T-cell help, whereas gene gun deliveries bias responses towards type 2 T-cell help (Feltquate et al., 1997; Pertmer, Roberts, and Haynes, 1996). DNAs injected in saline rapidly spread throughout the body. DNAs delivered by the gun are more localized at the target site. Following either method of inoculation, extracellular plasmid DNA has a short half life on the order of 10 minutes (Kawabata, Takakura, and Hashida, 1995; Lew et al., 1995). Vaccination by saline injections can be intramuscular (i.m.) or intradermal (i.d.) (Fynan et al., 1993).

Although intravenous and subcutaneous injections have met with different degrees of success for different plasmids (Bohm et al., 1998; Fynan et al., 1993), intraperitoneal injections have not met with success (Bohm et al., 1998; Fynan et al., 1993). Gene gun deliveries can be administered to the skin or to surgically exposed muscle. Methods and routes of DNA delivery that are effective at raising immune responses in mice are effective in other species.

Immunization by mucosal delivery of DNA has been less successful than immunizations using parenteral routes of inoculation. Intranasal administration of DNA in saline has met with both good (Asakura et al., 1997; Sasaki et al., 1998b) and limited (Fynan et al., 1993) success. The gene gun has successfully raised IgG following the delivery of DNA to the vaginal mucosa (Livingston et al., 1995). Some success at delivering DNA to mucosal surfaces has also been achieved using liposomes (McCluskie et al., 1998), microspheres (Chen et al., 1998a; Jones et al., 1997) and recombinant Shigella vectors (Sizemore, Branstrom, and Sadoff, 1995; Sizemore, Branstrom, and Sadoff, 1997).

The dose of DNA needed to raise a response depends upon the method of delivery, the host, the vector, and the encoded antigen. The most profound effect is seen for the method of delivery. From 10  $\mu$ g to 1 mg of DNA is generally used for saline injections of DNA, whereas from 0.2  $\mu$ g to 20  $\mu$ g of DNA is used for gene gun deliveries of DNA. In general, lower doses of DNA are used in mice (10-100  $\mu$ g for saline injections and 0.2  $\mu$ g to 2  $\mu$ g for gene gun deliveries), and higher doses in primates (100  $\mu$ g to 1 mg for saline injections and 2  $\mu$ g to 20  $\mu$ g for gene gun deliveries). The much lower amount of DNA required for gene gun deliveries reflect the gold beads directly delivering DNA into cells.

An example of the marked effect of an antigen on the raised response can be found in studies comparing the ability to raise antibody responses in rabbits of DNAs expressing the influenza hemagglutinin or an immunodeficiency virus envelope glycoprotein (Env) (Richmond et al., 1998). Under similar immunization conditions, the hemagglutinin-expressing DNA raised long lasting, high avidity, high titer antibody (~100  $\mu$ g per ml of specific antibody), whereas the Env-expressing DNA raised only transient, low avidity, and low titer antibody responses (<10  $\mu$ g per ml of specific antibody). These differences in raised antibody were hypothesized to reflect the hemagglutinin being a T-dependent antigen and the highly glycosylated immunodeficiency virus Env behaving as a T-independent antigen.

Both protein and recombinant viruses have been used to boost DNA-primed immune responses. Protein boosts have been used to increase neutralizing antibody responses to the HIV-1 Env. Recombinant pox virus boosts have been used to increase both humoral and cellular immune responses.

For weak immunogens, such as the immunodeficiency virus Env, for which DNA-raised antibody responses are only a fraction of those in naturally infected animals, protein boosts have provided a means of increasing low titer antibody responses (Letvin et al., 1997; Richmond et al., 1998). In a study in rabbits, the protein boost increased both the titers of antibody and the avidity and the persistence of the antibody response (Richmond et al., 1998). Consistent with a secondary immune response to the protein boost, DNA primed animals showed both more rapid increases in antibody, and higher titers of antibody following a protein boost than animals receiving only the protein. However, by a second protein immunization, the kinetics and the titer of the antibody response were similar in animals that had, and had not, received DNA priming immunizations.

Recombinant pox virus boosts have proved to be a highly successful method of boosting DNA-primed CD8+ cell responses (Hanke et al., 1998a; Kent et al., 1998; Schneider et al., 1998). Following pox virus boosters, antigen-specific CD8+ cells have been increased by as much as 10-fold in DNA primed mice or macaques. Studies testing the order of immunizations reveal that the DNA must be delivered first (Schneider et al., 1998). This has been hypothesized to reflect the DNA focusing the immune response on the desired immu-

nogens. The larger increases in CD8+ cell responses following pox virus boosts has been hypothesized to reflect both the larger amount of antigen expressed by the pox virus vector, as well as pox virus-induced cytokines augmenting immune responses (Kent et al., 1998; Schneider et al., 1998).

A number of different pox viruses can be used for the pox boost. A vaccinia virus termed modified vaccinia Ankara (MVA) has been particularly effective in mouse models (Schneider et al., 1998). This may reflect MVA, which is replication defective in mammalian models, being attenuated for the ability to evade host immune responses.

Responses raised by a DNA prime followed by pox virus boost can be highly effective at raising protective cell-mediated immune responses. In mice, intramuscular injections of DNA followed by recombinant pox boosts have protected against a malaria challenge (Schneider et al., 1998). In macaques, intradermal, but not gene gun DNA primes, followed by recombinant pox virus boosters have contained challenges with chimeras of simian and human immunodeficiency viruses (Robinson et al., 1999).

DNA vaccines for immunodeficiency viruses such as HIV-1 encounter the challenge of sufficiently limiting an incoming infection such that the inexorable long-term infections that lead to AIDS are prevented. Complicating this is that neutralizing antibodies is both difficult to raise and specific against particular viral strains (Burton and Montefiori, 1997; Moore and Ho, 1995). Given the problems with raising neutralizing antibody, much effort has focused on raising cell-mediated responses of sufficient strength to severely curtail infections. To date, the best success at raising high titers of Tc have come from immunization protocols using DNA primes followed by recombinant pox virus boosters. The efficacy of this protocol has been evaluated by determining the level of specific Tc using assays for cytolytic activity (Kent et al., 1998), by staining with MHC-specific tetramers for specific SIV Gag epitopes and by challenge with SIVs or SHIVs (Hanke, 1999).

A number of salient findings are emerging from preclinical trials using DNA primes and recombinant pox virus boosts. The first is that challenge infections can be contained below the level that can be detected using quantitative RT-PCR analyses for plasma viral RNA (Robinson et al., 1999). The second is that this protection is long lasting and does not require the presence of neutralizing antibody (Robinson et al., 1999). The third is that intradermal DNA priming with saline injections of DNA is superior to gene gun priming for raising protective immunity ( $P=0.01$ , Fisher's exact test) (Robinson et al., 1999).

The novel pGA vectors of the present invention have a prokaryotic origin of replication, a selective marker gene for plasmid selection, and a transcription cassette for eukaryotic cells. Unique to the pGA vectors of the present invention is the inclusion of the lambda terminator in the same transcriptional orientation, and following, the selective marker gene. This terminator sequence prevents read-through from the kanamycin cassette into vaccine sequences while the plasmid is being produced in bacteria. Prevention of transcriptional read-through stabilizes vaccine insert sequences by limiting the exposure of secondary structures that can be recognized by bacterial endonucleases.

A transcription cassette as incorporated in the pGA vectors of the present invention uses sequences from the cytomegalovirus immediate early promoter (CMVIE) and from the bovine growth hormone polyadenylation sequences (BGHpA) to control transcription. A leader sequence that is a synthetic homolog of the tissue plasminogen activator gene leader sequence (tPA) is optional in the transcription cassette.

The vectors of the present invention differ in the sites that can be used for accepting vaccine inserts and in whether the transcription cassette includes intron A sequences in the CMVIE promoter. Both intron A and the tPA leader sequence have been shown in certain instances to supply a strong expression advantage to vaccine inserts (Chapman et al., 1991).

pGA1 is a 3894 bp plasmid. pGA1 comprises a promoter (bp 1-690), the CMV-intron A (bp 691-1638), a synthetic mimic of the tPA leader sequence (bp 1659-1721), the bovine growth hormone polyadenylation sequence (bp 1761-1983), the lambda T0 terminator (bp 1984-2018), the kanamycin resistance gene (bp 2037-2830) and the ColEI replicator (bp 2831-3890). The DNA sequence of the pGA1 construct (SEQ ID NO: 1) is shown in FIG. 2. In FIG. 1, the indicated restriction sites are single cutters useful for the cloning of vaccine inserts. The ClaI or BspDI sites are used when the 5' end of a vaccine insert is cloned upstream of the tPA leader. The NheI site is used for cloning a sequence in frame with the tPA leader sequence. The sites listed between SmaI and BlnI are used for cloning the 3' terminus of a vaccine insert.

pGA2 is a 2947 bp plasmid lacking the 947 bp of intron A sequences found in pGA1. pGA2 is the same as pGA1, except for the deletion of intron A sequences. pGA2 is valuable for cloning sequences which do not require an upstream intron for efficient expression, or for cloning sequences in which an upstream intron might interfere with the pattern of splicing needed for good expression. FIG. 3 presents a map of pGA2 with useful restriction sites for cloning vaccine inserts, and FIG. 4 shows the DNA sequence SEQ ID NO: 2. The use of restriction sites for cloning vaccine inserts into pGA2 is the same as that used for cloning fragments into pGA1.

pGA3 is a 3893 bp plasmid that contains intron A. pGA3 is the same as pGA1 except for the cloning sites that can be used for the introduction of vaccine inserts. In pGA3, inserts cloned upstream of the tPA leader sequence use a Hind III site. Sequences cloned downstream from the tPA leader sequence use sites between the SmaI and the BlnI site. In pGA3, these sites include a BamHI site. FIG. 5 shows the map for pGA3, and FIG. 6 shows the DNA sequence SEQ ID NO: 3.

In view of the teachings herein, one skilled in the art will recognize that any vaccine insert known in the art can be used in the novel pGA constructs described herein, including but not limited to viral pathogens like HIV, influenza, measles, herpes, Ebola, and the like.

For example, the present invention contemplates inserts from immunodeficiency virus, more preferably HIV, including all clades of HIV-1 and HIV-2 and modifications thereof; influenza virus genes including all subtypes and modifications thereof; and vaccine inserts derived from measles genes. One skilled in the art will appreciate that the discussion about inserts derived from immunodeficiency virus; influenza virus; measles virus; and modifications thereof are exemplary in nature and provided for the sake of illustration only.

The immunodeficiency virus vaccine inserts of the present invention were designed to express non-infectious virus like particles (VLPs) from a single DNA. This was achieved using the subgenomic splicing elements normally used by immunodeficiency viruses to express multiple gene products from a single viral RNA. Important to the subgenomic splicing patterns are (i) splice sites and acceptors present in full length viral RNA, (ii) the Rev responsive element (RRE) and (iii) the Rev protein. The splice sites in retroviral RNAs use the canonical sequences for splice sites in eukaryotic RNAs. The RRE is an ~200 bp RNA structure that interacts with the Rev protein to allow transport of viral RNAs from the nucleus to



the cytoplasm. In the absence of Rev, the ~10 kb RNA of immunodeficiency virus undergoes splicing to the mRNAs for the regulatory genes Tat, Rev, and Nef. These genes are encoded by exons present between RT and Env and at the 3' end of the genome. In the presence of Rev, the singly spliced mRNA for Env and the unspliced mRNA for Gag and Pol are expressed in addition to the multiply spliced mRNAs for Tat, Rev, and Nef.

The expression of non-infectious VLPs from a single DNA affords a number of advantageous features to an immunodeficiency virus vaccine. The expression of a number of proteins from a single DNA affords the vaccinated host the opportunity to respond to the breadth of T- and B-cell epitopes encompassed in these proteins. The expression of proteins containing multiple epitopes affords the opportunity for the presentation of epitopes by diverse histocompatibility types. By using whole proteins, one offers hosts of different histocompatibility types the opportunity to raise broad-based T-cell responses. Such may be essential for the effective containment of immunodeficiency virus infections, whose high mutation rate supports ready escape from immune responses (Evans et al., 1999) (Poignard et al., 1999, Evans, et al., 1995). Just as in drug therapy, multi-epitope T-cell responses that require multiple mutations for escape will provide better protection than single epitope T-cell responses that require only a single mutation for escape.

Antibody responses are often best primed by multi-valent vaccines that present an ordered array of an epitope to responding B-cells (Bachmann, Zinkernagel, 1997). Virus-like particles, by virtue of the multivalency of Env in the virion membrane, will facilitate the raising of anti-Env antibody responses. These particles will also present non-denatured and normal forms of Env to the immune system.

The novel vectors of the present invention can be administered to a patient in the presence of adjuvants or other substances that have the capability of promoting DNA uptake or recruiting immune system cells to the site of the inoculation. Embodiments include combining the DNA vaccine with conventional adjuvants or genetic adjuvants. Conventional adjuvants, including reagents that favor the stability and uptake of the DNA, recruit immune system cells to the site of inoculation, or facilitate the immune activation of responding lymphoid cells, include but are not limited to oil and water emulsions (for example, complete Freund's adjuvant and incomplete Freund's adjuvant), *Corynebacterium parvum*, *Bacillus Calmette Guérin*, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, "REGRESSIN" (Vetrepharm, Athens, Ga.), "AVRIDINE" (N,N-di-octadecyl-N', N'-bis(2-hydroxyethyl)-propanediamine), paraffin oil, and muramyl dipeptide. The present invention also contemplates the use of genetic adjuvants such as immunomodulatory molecules encoded in a co-inoculated DNA. The co-inoculated DNA can be in the same vaccine construct as the vaccine immunogen or in a separate DNA vector.

A vaccine according to the present invention can be administered in a variety of ways including through any parenteral or topical route. For example, an individual can be inoculated by intravenous, intraperitoneal, intradermal, subcutaneous or intramuscular methods. Inoculation can be, for example, with a hypodermic needle, needleless delivery devices such as those that propel a stream of liquid into the target site, or with the use of a gene gun that bombards DNA on gold beads into the target site. The vector comprising the pathogen vaccine insert can be administered to a mucosal surface by a variety of methods including intranasal administration, i.e., nose drops

or inhalants, or intrarectal or intravaginal administration by solutions, gels, foams, or suppositories. Alternatively, the vector comprising the vaccine insert can be orally administered in the form of a tablet, capsule, chewable tablet, syrup, emulsion, or the like. In an alternate embodiment, vectors can be administered transdermally, by passive skin patches, iontophoretic means, and the like.

Any appropriate physiologically acceptable medium is suitable for introducing the vector comprising the pathogen vaccine insert into the patient. For example, suitable pharmaceutically acceptable carriers known in the art include, but are not limited to, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

The present invention is further illustrated by the following examples, which are provided by way of illustration and should not be construed as limiting. The contents of all references, published patents and patents cited throughout the present application are hereby incorporated by reference in their entirety.

#### EXAMPLE 1

##### Structure and Sequence of PGA1

pGA1 as illustrated in FIG. 1 and FIG. 2 contains the ColE1 origin of replication, the kanamycin resistance gene for antibiotic selection, the lambda T0 terminator, and a eukaryotic expression cassette including an upstream intron. The ColE1 origin of replication is a 600 nucleotide DNA fragment that contains the origin of replication (ori), encodes an RNA primer, and encodes two negative regulators of replication initiation. All enzymatic functions for replication of the plasmid are provided by the bacterial host. The original constructed plasmid that contained the ColE1 replicator was pBR322 (Bolivar, et al. 1977; Sutcliffe, et al. 1978).

The kanamycin resistance gene is an antibiotic resistance gene for plasmid selection in bacteria. The lambda T0 terminator prevents read through from the kanamycin resistance gene into the vaccine transcription cassette during prokaryotic growth of the plasmid (Scholtissek and Grosse, 1987). By preventing read through into the vaccine expression cassette, the terminator helps stabilize plasmid inserts during growth in bacteria.

The eukaryotic expression cassette is comprised of the CMV immediate early promoter including intron A (CMVIE-IA) and termination sequences from the bovine growth hormone polyadenylation sequence (BGHPA). A synthetic mimic of the leader sequence for tissue plasminogen activator (tPA) is included as an option within the transcription cassette. Cassettes with these elements have proven to be highly effective for expressing foreign genes in eukaryotic cells (Chapman et al., 1991). Cloning sites within the transcription cassette include a ClaI site upstream of the tPA leader, a NheI site for cloning in frame with the tPA leader, and XmnI, SmaI, RsrII, AvrII, and BlnI sites for cloning prior to the BGHPA.

The ColE1 replicator, the Kanamycin resistance gene and transcriptional control elements for eukaryotic cells were combined in one plasmid using polymerase chain reaction (PCR) fragments from a commercial vector, pZER0-2 (Invitrogen, Carlsbad, Calif.) and a eukaryotic expression vector, pJW4303 (Lu et al., 1997).

A 1853 bp fragment from pZER02 from nt 1319 to nt 3178 included the ColE1 origin of replication and the kanamycin



## 19

resistance gene. A 2040 bp fragment from pJW4303 from nt 376 to nt 2416 included the CMVIE promoter with intron A, a synthetic homolog of the tissue plasminogen activator leader (tPA), and the bovine growth hormone polyadenylation site (BGHpA). Fragments were amplified by polymerase chain reaction (PCR) with oligonucleotide primers containing SalI sites. A ligation product with the transcription cassettes for Kanamycin resistance from pZeRO2 and the eukaryotic transcription cassette from pJW4303 in opposite transcriptional orientations was identified for further development. Nucleotide numbering for this parent for the pGA vectors was started from the first bp of the 5' end of the CMV promoter.

The T0 terminator was introduced into this parent for the pGA vectors by PCR amplification of a 391 bp fragment with a BamH I restriction endonuclease site at its 5' end and an Xba I restriction endonuclease site at its 3' end. The initial 355 bp of the fragment were sequences in the BGHpA sequence derived from the pJW4303 transcription cassette, the next 36 bases in a synthetic oligonucleotide introduced the T0 sequence and the Xba I site. The introduced T0 terminator sequences comprised the sequence:

(SEQ ID NO: 6)  
5' - ATAAAAACGCCGCGCGCAACCGAGCGTTCGTGAA - 3'.

The T0 terminator containing BamH1-XbaI fragment was substituted for the homologous fragment without the T0 terminator in the plasmid created from pXeRO 2 and pJW4303. The product was sequenced to verify the TO orientation.

A region in the eukaryotic transcription cassette between nucleotides 1755-1845 contained the last 30 bp of the reading frame for SIV nef. This region was removed from pGA by mutating the sequence at nt1858 and generating an Avr II restriction endonuclease site. A naturally occurring Avr II site is located at nt1755. Digestion with Avr II enzyme and then religation with T4 DNA ligase allowed for removal of the SIV segment of DNA between nucleotides 1755-1845. To facilitate cloning of HIV-1 sequences, into pGA vectors a ClaI site was introduced at bp1645 and an RsrII site at bp 1743 using site directed mutagenesis. Constructions were verified by sequence analyses.

## EXAMPLE 2

## Structure and Sequence of PGA2

pGA2, as illustrated in FIG. 3, and FIG. 4, is identical to pGA1 except for deletion of the intron A sequence from the CMVIE promoter. pGA2 was created from pGA1 by introducing a Cla I site 8 bp by downstream from the mRNA cap site in the CMVIE promoter. The Cla I site was introduced using oligonucleotide-directed mutagenesis using the complementary primers

(SEQ ID NO: 7)  
5' - CCGTCAGATCGCATCGATACGCCATCCACG - 3'  
and

(SEQ ID NO: 8)  
5' - CGTGGATGGCGTATCGATGCGATCTGACGG - 3'.

After insertion of the new Cla I site, pGA1 was digested with Cla I to remove the 946 bp Cla I fragment from pGA1, and then religated to yield pGA2.

## 20

## EXAMPLE 3

## Structure and Sequence of PGA3

pGA3 as shown in FIG. 5 and FIG. 6 is identical to pGA1 except for the introduction of a HindIII site in stead of the ClaI site at nt 1645 and a BamHI site instead of the RsrII site at nucleotide 1743.

## EXAMPLE 4

## Comparative Expression and Immunogenicity of PGA3 and pJW4303

To determine the efficacy of the pGA plasmids as vaccine vectors, a pGA plasmid was compared to the previously described vaccine vector pJW4303. The pJW4303 plasmid has been used for DNA vaccinations in mice, rabbits, and rhesus macaques (Robinson et al. 1999; Robinson et al., 1997; Pertmer, et al., 1995; Feltquate, et al. 1997; Torres, et al. 1999). Comparisons were done with pGA3 with a vaccine insert encoding the normal, plasma-membrane form of the A/PR/8/34 (H1N1) influenza virus hemagglutinin (pGA3/H1) and pJW4303 encoding the same fragment (pJW4303/H1). Both pGA3 and pJW4303 contain intron A upstream of influenza H1 sequences.

The pGA3/H1 and pJW4303/H1 vaccine plasmids expressed similar levels of H1 in eukaryotic cells, as summarized below:

TABLE 5

In Vitro Expression Levels of HA plasmids.

Plasmids	Relative HA Units	
	Supernatant	Cell Lysate
PGA3/H1	0.1 ± 0.1	5.7 ± 0.6
pGA vector	0.0 ± 0.0	0.2 ± 0.1
pJW4303/H1	0.3 ± 0.05	4.8 ± 0.5
pJW4303	0.0 ± 0.0	0.1 ± 0.1

Human embryonic kidney 293T cells were transiently transfected with 2 µg of plasmid and the supernatants and cell lysates assayed for H1 using an antigen-capture ELISA. The capture antibody was a polyclonal rabbit serum against H1, and the detection antibody, polyclonal mouse sera against H1. pGA3/H1 expressed slightly more H1 than pJW4303/H1 (5.8 HA units as opposed to 5.1 H1 units (Table 6). As expected, 90% of the H1 antigen was in the cell lysates. A comparative immunization study using pGA3/H1, and pJW4303/H1 demonstrated comparable or better immunogenicity for pGA3/H1 than pJW4303/H1 (FIG. 7). Immunogenicity was assessed in BALB/c mice. In this example, mice were vaccinated with DNA coated gold particles via gene gun. Mice were primed and boosted with a low dose (0.1 µg) or a high dose (1 µg) of the plasmid DNAs. The booster immunization was given at 4 weeks after the priming immunization. The amount of anti-H1 IgG raised in response to immunizations was as high or higher following immunization with pGA3/H1 than following immunization with pJW4303/H1 (FIG. 7). Thus the pGA vector proved to be as effective, or more effective, than the pJW4303 vector at raising immune responses.

## 21

## EXAMPLE 5

## Immunodeficiency Virus Vaccine Inserts in pGA Vectors

Immunodeficiency virus vaccine inserts expressing virus like particles have been developed in pGA1 and pGA2. The VLP insert was designed with clade B HIV-1 sequences so that it would match HIV-1 sequences that are endemic in the United States. Within clade B, different isolates exhibit clustal diversity, with each isolate having overall similar diversity from the consensus sequence for the clade (Subbarao, Schochetman, 1996). Thus, any clade B isolate can be

## 22

the CCR-5-using VLP (pGA2/JS2), a derivative of JS2 was prepared that expresses an Env-defective VLP. This plasmid insert was designated JS5. Although it is anticipated that this sequence will be a less effective vaccine than the Env-containing JS2 VLP, the non-Env containing VLP offers certain advantages for vaccination. These include the ability to monitor vaccinated populations for infection by sero-conversion to Env. Deletion of Env sequences also reduces the size of the vaccine plasmid. The DNA sequence of pGA2/JS2 is shown in FIG. 17 and that of pGA1/JS5 in FIG. 18.

To achieve a VLP plasmid with high expression, candidate vaccines were constructed from 7 different HIV-1 sequences, as shown in the following table:

TABLE I

Comparison of candidate vaccine inserts					
Plasmid designation	Sequences tested	Ability to grow plasmid	Expression of Gag	Expression of Env	Comment
BH10-VLP	BH10	good	good	good	X4 Env
6A-VLP	6A env in BH10-VLP	poor	not tested	not tested	
BAL-VLP	BAL env in BH10-VLP	good	poor	poor	
ADA-VLP	ADA env in BH10-VLP	good	good	good	chosen for vaccine, renamed pGA1/JS1
CDC-A-VLP	CDC-A env in BH10-VLP	good	good	poor	
CDC-B-VLP	CDC-B-env in BH10-VLP	good	good	good	not as favorable expression as ADA
CDC-C-VLP	CDC-C env in BH10-VLP	good	good	good	not as favorable expression as ADA

used as a representative sequence for other clade B isolates. HIV-1 isolates use different chemokine receptors as co-receptors. The vast majority of viruses that are undergoing transmission use the CCR-5 co-receptor (Berger, E. A., 1997). Therefore the vaccine insert was designed to have a CCR-5 using Env.

The expression of VLPs with an R5-Env by a HIV-1 DNA vaccine also has the advantage of supporting Env-mediated entry of particles into professional antigen presenting cells (APCs) such as dendritic cells and macrophages. Both dendritic cells and macrophages express the CD4 receptor and the CCR-5 co-receptor used by CCR-5-tropic (R5) HIV-1 Envs. By using an R5 Env in the vaccine, the VLP expressed in a transfected non-professional APC (for example keratinocyte or muscle cells) can gain entry into the cytoplasm of an APC by Env-mediated entry. Following entry into the cytoplasm of the APC, the VLP will be available for processing and presentation by class I histocompatibility antigens. DNA-based immunizations rely on professional APCs for antigen presentation (Corr et al., 1996; Fu, et al., 1997; Iwasaki A, et al., 1997). Much of DNA-based immunization is accomplished by direct transfection of professional APC (Condon et al., 1996; Porgador et al., 1998). Transfected muscle cells or keratinocytes serve as factories of antigen but do not directly raise immune response (Torres et al., 1997). By using an expressed antigen that is assembled and released from transfected keratinocytes or muscle cells and then actively enters professional APC, the efficiency of the immunization may be increased.

Goals in the construction of pGA2/JS2 were (i) to achieve a CCR-5-using clade B VLP with high expression, (ii) to produce a VLP that was non infectious and (iii) to minimize the size of the vaccine plasmid. Following the construction of

An initial construct, pBH10-VLP, was prepared from IIIb sequences that are stable in bacteria and have high expression in eukaryotic cells. The BH10 sequences were obtained from the NIH-sponsored AIDS Repository (catalog #90). The parental pBH10 was used as the template for PCR reactions to construct pBH10-VLP.

Primers were designed to yield a Gag-Rt PCR product (5' PCR product) encompassing from 5' to 3' 105 by of the 5' untranslated leader sequence and gag and pol sequences from the start codon for Gag to the end of the RT coding sequence. The oligonucleotide primers introduced a ClaI site at the 5' end of the PCR product and EcoRI and NheI sites at the 3' end of the PCR product. Sense primer 1 (5'-GAGCTCTATCGATGCAGGACTCGGCTTGC-3' (SEQ ID NO: 9)) and antisense primer 2 (5'-GGCAGGTTTAAATCGCTAGCCTATGCTCTCC-3' (SEQ ID NO: 10)) were used to amplify the 5' PCR product.

The PCR product for the env region of HIV-1 (3' PCR product) encompassed the vpu, tat, rev, and env sequences and the splice acceptor sites necessary for proper processing and expression of their respective mRNAs. An EcoRI site was introduced at the 5' end of this product and NheI and RsrII sites were introduced into the 3' end. Sense primer 3 (5'-GGGCAGGAGTGCTAGCC-3' (SEQ ID NO: 11)) and antisense primer 4 (5'-CCACACTACTTTCGGACCGCTAGC-CACCC-3' (SEQ ID NO: 12)) were used to amplify the 3' PCR product.

The 5' PCR product was cloned into pGA1 at the ClaI and NheI sites and the identity of the construct confirmed by sequencing. The 3' PCR product was then inserted into the 5' clone at the EcoRI and NheI sites to yield pBH10-VLP. The construction of this VLP resulted in proviral sequences that lacked LTRs, integrase, vif, and vpr sequences (see FIG. 8A).

23

Because the BH10-VLP had an X4 rather than an R5 Env, sequences encoding six different R5 Envs were substituted for env sequences in BH10-VLP. This was done by cloning EcoRI to BamHI fragments encompassing tat, rev, vpu and env coding sequences from different viral genomes into pBH10-VLP. The resulting env and rev sequences were chimeras for the substituted sequences and BH10 sequences (for example see FIG. 8B). In the case of the ADA envelope, a BamHI site was introduced into the ADA sequence to facilitate substituting an EcoRI to BamHI fragment for the EcoRI to BamHI region of the BH10-VLP (FIG. 8). The results of these constructions are summarized in Table 1. Of the six sequences tested, one, the 6A-VLP was found to be associated with poor plasmid growth in transformed bacteria. This plasmid was not used for further vaccine development (Table 1).

Among the plasmids exhibiting good growth in bacteria, the best expression of the VLP was found for the ADA-VLP

24

(Table 1). In transient transfections in 293T cells, the expression of the ADA-VLP was higher than that of wt proviruses for ADA or IIIb (FIG. 9). Expression was also higher than for a previous VLP-vaccine (dpol) (Richmond et al., 1998) that had successfully primed cytotoxic T-cell (Tc) responses in rhesus macaques (Kent et al., 1998).

## EXAMPLE 6

## Safety Mutations

Once the ADA-VLP had been identified as a favorable candidate for further vaccine development, this plasmid was mutated to increase its safety for use in humans. Further mutations disabled the Zinc fingers in NC that are active in the encapsidation of viral RNA, and added point mutations to inactivate the activity of the viral reverse transcriptase and the viral protease (FIG. 8). The following table summarizes the location of the safety point mutations

TABLE 2

Location of safety point mutations in pGA/JS2 and pGA/JS5 to inhibit viral RNA packaging and abolish reverse transcriptase activity in vaccine constructs				
GENE	REGION	FUNCTION	AMINO ACID CHANGE <sup>1</sup>	LOCATION <sup>2</sup>
Gag	Zn finger	Viral RNA packaging	C392S	1285/1287
Gag	Zn finger	Viral RNA packaging	C395S	1294/1296
Gag	Zn finger	Viral RNA packaging	C413S	1348/1350
Gag	Zn finger	Viral RNA packaging	C416S	1357/1359
Pol	RT	Polymerase activity	D185N	2460/2462
Pol	RT	Strand transfer	W266T	2703/2704/2705
Pol	RNase H	RNase activity	E478Q	3339

<sup>1</sup>Amino acid number corresponds to individual genes in HIV-1 BH10 sequence;

<sup>2</sup>Nucleotide number in wt HIV-1 BH10 sequence

The mutations were made using a site directed mutagenesis kit (Stratagene) following the manufacturer's protocol. All mutations were confirmed by sequencing. Primer pairs used for the mutagenesis were:

- (A) C15S ZN1  
5'-GGTTAAGAGCTTCAATAGCGGCAAGAAGGGC-3' (SEQ ID NO: 13)  
C15S ZN2  
5'-GCCCTTCTTTGCCGCTATTGAAGCTCTTAACC-3' (SEQ ID NO: 14)
- (B) C36S ZN3  
5'-GGGCAGCTGGAAAAGCGGAAAGGAAGG-3' (SEQ ID NO: 15)  
C36S ZN4  
5'-CCTTCCTTTCCGCTTTTCCAGCTGCCC-3' (SEQ ID NO: 16)
- (C) D185N RT1  
5'-CCAGACATAGTTATCTATCAATACATGAACGATTTGTATGTAGG-3' (SEQ ID NO: 17)  
D185N RT2  
5'-CCTACATACAAATCGTTTCATGTATTGATAGATAACTATGTCTGG-3' (SEQ ID NO: 18)
- (D) W266T RT3  
5'-GGGGAAATTGAATACCGCAAGTCAGATTTACCC-3' (SEQ ID NO: 19)  
W266T RT4  
5'GGTAAATCTGACTTGCAGTATTCAATTTCCCC-3' (SEQ ID NO: 20)
- (E) E478Q RT5  
5'-CCCTAACTAACACAAATCAGAAAACCTCAGTTACAAGC-3' (SEQ ID NO: 21)  
E478Q RT6  
5'-GCTTGTAAGTGGTTTCTGATTTGTTGTGTAGTTAGGG-3' (SEQ ID NO: 22)
- (F) D25A Prt1  
5'-GGCAACTAAGGAAGCTCTATTAGCCACAGGAGC-3' (SEQ ID NO: 23)  
D25A prt2  
5'-GCTCCTGTGGCTAATAGAGCTTCCTTTAGTTGCC-3' (SEQ ID NO: 24)

## 25

The ADA-VLP with the zinc finger and RT mutations was found to express Gag and Env more effectively than the VLP plasmid without the mutations (FIG. 10). The mutation that inactivated the protease gene markedly reduced VLP expression (FIG. 10) and was not included in the further development of the vaccine plasmid. The ADA-VLP without mutations was designated JS 1 and the ADA-VLP with mutations, JS2.

## EXAMPLE 7

## Construction of the JS5 Vaccine Insert

The JS5 insert, a plasmid expressing Gag, RT, Tat, and Rev was constructed from JS2 by deleting a BglII fragment in the ADA Env (FIG. 8). This deletion removed sequences from nt 4906-5486 of the pGA2/JS2 sequence and results in a premature stop codon in the env gene leading to 269 out of the 854 amino acids of Env being expressed while leaving the tat, rev, and vpu coding regions the RRE and splice acceptor sites intact. The DNA sequence of pGA1/JS5 is shown in FIG. 18.

## EXAMPLE 8

## Minimizing the Size of the JS2 and JS5 Vaccine Plasmids

The JS2 and JS5 vaccine inserts were originally constructed in pGA1, a vector that contains the ~1 kb intron A of the CMVIE promoter upstream of the vaccine insert. To determine whether this intron was necessary for high levels of vaccine expression, pGA2 vectors lacking intron A were constructed expressing the JS2 and JS5 vaccine inserts. In expression tests, pGA2 proved to have as good an expression pattern as pGA1 for JS2 (FIG. 11). In contrast to this result, JS5 was expressed much more effectively by pGA1 than pGA2 (FIG. 11). For the JS5 insert, the absence of intron A resulted in 2-3-fold lower levels of expression than in the presence of intron A.

## EXAMPLE 9

## Testing for the Efficacy of the Safety Mutations in the Vaccine Inserts JS2 and JS5

The three point mutations in RT (Table 2) completely abolished detectable levels of reverse transcriptase activity for JS2 and JS5. A highly sensitive reverse transcriptase assay was used in which the product of reverse transcription was amplified by PCR (Yamamoto, Folks, Heneine, 1996). This assay can detect reverse transcriptase in as few as 10 viral particles. Reverse transcriptase assays were conducted on the culture supernatants of transiently transfected cells. Reverse transcriptase activity was readily detected for as few as 10 particles ( $4 \times 10^{-3}$  pg of p 24) in the JS 1 vaccine but could not be detected for the JS2 or JS5 inserts.

The deletions and zinc finger mutations in the JS2 and JS5 vaccine inserts (Table 2) reduced the levels of viral RNA in particles by at least 1000-fold. Particles pelleted from the supernatants of transiently transfected cells were tested for the efficiency of the packaging of viral RNA. The VLPs were treated with DNase, RNA was extracted and the amount of RNA standardized by p24 levels before RT PCR. The RT PCR reaction was followed by nested PCR using primers specific for viral sequences. End point dilution of the VLP RNA was compared to the signal obtained from RNA packaged in wt HIV-1 Bal virus.

## 26

Packaging for both JS2 and JS5 was restricted by the deletions in the plasmid by 500-1000-fold, as summarized below:

TABLE 3

Packaging of viral RNA is reduced in pGA2/JS2 and pGA1/JS5 VLPs		
Vaccine Construct	Deletions/Mutations	Copies vRNA relative to wt HIV-1 bal
HIV-1 bal	Wt	1
pGA1/JS1 VLP	Deleted: LTRs, int, vif, vpr, nef	.002
pGA1/JS2 VLP	Deleted: LTRs, int, vif, vpr, nef; Mutations in Zn fingers and RT	.0001
pGA1/JS4 VLP	Deleted: LTRs, int, vif, vpr, nef	.001
pGA1/JS5 VLP	Deleted: LTRs, int, vif, vpr, nef, env; Mutations in Zn fingers and RT	.001

The zinc finger mutations decreased the efficiency of packaging for the JS2 particles a further 20-fold but did not further affect the efficiency of packaging for the JS5 particles. This pattern of packaging was reproducible for particles produced in independent transfections.

## EXAMPLE 10

## Western Blot Analyses of Protein Expression

Western blot analyses, shown in FIGS. 12A-D, revealed the expected patterns of expression of pGA2/JS2 and pGA1/JS5. Both immature and mature proteins were observed in cell lysates, whereas only the mature forms of Gag and Env were found in the VLP-containing lysates (FIGS. 12B and 12C). Reverse transcriptase was readily detected in cell lysates (FIG. 12D).

## EXAMPLE 11

## pGA2/89.6 SHIV Vector Construction

Initial immunogenicity trials have been conducted with a SHIV-expressing VLP rather than the HIV-1-expressing vaccine plasmids. SHIVs are hybrids of simian and human immunodeficiency virus sequences that grow well in macaques (Li et al., 1992). By using a SHIV, vaccines that are at least partially of HIV-1 origin can be tested for efficacy in macaque models.

pGA2/89.6 (also designated as pGA2/M2) expresses sequences from SHIV-89.6 (Reimann, Li, Voss, et al., 1996; Reimann, Li, Veazey, et al., 1996). The 89.6 Env represents a patient isolate (Collman et al., 1992). The SHIV-89.6 virus is available as a highly pathogenic challenge stock, designated SHIV-89.6P (Reimann, Li, Voss, et al., 1996; Reimann, Li, Veazey, et al., 1996), which allows a rapid determination of vaccine efficacy. The SHIV-89.6P challenge can be administered via both intrarectal and intravenous routes. SHIV-89.6 and SHIV-89.6P do not generate cross-neutralizing antibody.

pGA2/89.6 (FIG. 13) has many of the design features of pGA2/JS2. Both express immunodeficiency virus VLPs: HIV-1 VLP in the case of pGA2/JS2, while the VLP expressed by pGA2/89.6 is a SHIV VLP. The gag-pol sequences in pGA2/89.6 are from SIV239, while the tat, rev, and env sequences are from HIV-1-89.6. pGA2/89.6 also differs from pGA2/JS2 in that the integrase, vif and vpr sequences have not been deleted, nor has the reverse tran-

scriptase gene been inactivated by point mutations. Finally, the zinc fingers in NC have been inactivated by a deletion and not by point mutations.

pGA1/Gag-Pol was also constructed to allow evaluation of the protective efficacy of a Gag-Pol expressing vector with the Gag-Pol-Env expressing pGA2/89.6. This vector was constructed from pGA1/JS5 and pGA2/89.6 (FIG. 13).

EXAMPLE 12

Comparison of the Expression of PGA2/89.6 SHIV Plasmid with pGA2/JS2 Expression

Both pGA2/89.6 and pGA1/Gag-Pol expressed similar levels of Gag as pGA2/JS2. Comparative studies for expression were performed on transiently transfected 293T cells. Analyses of the lysates and supernatants of transiently transfected cells revealed that both plasmids expressed similar

The vaccination trial compared i.d. and i.m. administration of the DNA vaccine and the ability of a genetic adjuvant, a plasmid expressing macaque GM-CSF, to enhance the immune response raised by the vaccine inserts. Vaccination was accomplished by priming with DNA at 0 and 8 weeks and boosting with rMVA at 24 weeks. For co-delivery of a plasmid expressing GM-CSF, 1-100 µl i.d. inoculation was given with a solution containing 2.5 mg of pGA2/89.6 and 2.5 mg per ml of pGM-CSF.

I.d. and i.m. deliveries of DNA were compared for two doses, 2.5 mg and 250 µg of DNA. Four vaccine groups of six rhesus macaques were primed with either 2.5 mg (high-dose) or 250 µg (low-dose) of DNA by intradermal (i.d.) or intramuscular (i.m.) routes using a needleless jet injection device (Bioject, Portland Oreg.). The 89.6-MVA booster immunization (2×10<sup>8</sup> pfu) was injected with a needle both i.d. and i.m. A control group included two mock immunized animals and two naive animals. The vaccination protocol is summarized as follows:

TABLE 4

Vaccination Trial				
Group, (# macaque)	Prime at 0 and 8 weeks	Immunogen	Boost at 24 weeks	Immunogen
1 (6)	i.d. bioject	2.5 mg VLP DNA	i.d. + i.m.	MVA gag-pol-env
2 (6)	i.m. bioject	2.5 mg VLP DNA	i.d. + i.m.	MVA gag-pol-env
3 (6)	i.d. bioject	250 µg VLP DNA	i.d. + i.m.	MVA gag-pol-env
4 (6)	i.m. bioject	250 µg VLP DNA	i.d. + i.m.	MVA gag-pol-env
5 (6)	i.d. bioject	2.5 mg gag-pol DNA	i.d. + i.m.	MVA gag-pol
6 (6)	i.d. bioject	250 µg gag-pol DNA	i.d. + i.m.	MVA gag-pol
7 (6)	i.d. bioject	250 µg VLP DNA + 250 µg GM-CSF DNA	i.d. + i.m.	MVA gag-pol-env
8 (5)	i.d. bioject i.d. + i.m. control MVA	2.5 mg control DNA control MVA	i.d. + i.m.	control MVA
9 (4)	i.d., bioject	250 µg control DNA + 250 µg GM-CSF DNA	i.d. + i.m.	MVA gag-pol-env
10 (6)	i.d. + i.m.	MVA gag-pol-env	i.d. + i.m.	MVA gag-pol-env

VLP DNA expresses all SHIV-89.6 proteins except Nef, truncated for LTRs, 2<sup>nd</sup> ZN++ finger, mutated to express cell surface Env; gag-pol DNA expresses SIV mac 239 gag-pol; MVA gag-pol-env expresses 89.6 truncated env and SIV mac 239 gag-pol; MVA gag-pol expresses SIVmac239 gag-pol; MVA dose is 1 × 10<sup>8</sup> pfu

levels of capsid antigen (FIG. 14). The capsid proteins were quantified using commercial antigen capture ELISA kits for HIV-1 p24 and SIV p27.

EXAMPLE 13

pGA2/89.6 SHIV Vaccine Protocol

A rhesus macaque model was used to investigate the ability of systemic DNA priming followed by a recombinant MVA (rMVA) booster to protect against a mucosal challenge with the SHIV-89.6P challenge strain (Amara et al, 2001).

The DNA component of the vaccine (pGA2/89.6) was made as described in Example 11 and expressed eight immunodeficiency virus proteins (SIV Gag, Pol, Vif, Vpx, and Vpr and HIV Env, Tat, and Rev) from a single transcript using the subgenomic splicing mechanisms of immunodeficiency viruses. The rMVA booster (89.6-MVA) was provided by Dr. Bernard Moss (NIH) and expresses both the HIV 89.6 Env and the SIV 239 Gag-Pol, inserted into deletion II and deletion III of MVA respectively, under the control of vaccinia virus early/late promoters (Wyatt and Moss, unpublished results). The 89.6 Env protein was truncated for the C-terminal 115 amino acids of gp41. The modified H5 promoter controlled the expression of both foreign genes.

Animals were challenged seven months after the rMVA booster to test whether the vaccine had generated long-term immunity. Because most HIV-1 infections are transmitted across mucosal surfaces, an intrarectal challenge was administered to test whether the vaccine could control a mucosal immunodeficiency virus challenge. Briefly, the challenge stock (5.7×10<sup>9</sup> copies of viral RNA per ml) was produced by one i.v. followed by one intrarectal passage in rhesus macaques of the original SHIV-89.6P stock. Lymphoid cells were harvested from the intrarectally infected animal at peak viremia, CD8-depleted and mitogen-stimulated for stock production. Prior to intrarectal challenge, fasted animals were anesthetized (ketamine, 10 mg/kg) and placed on their stomach with the pelvic region slightly elevated. A feeding tube [8Fr (2.7 mm)×16 inches (41 cm), Sherwood Medical, St. Louis, Mo.] was inserted into the rectum for a distance of 15-20 cm. Following insertion of the feeding tube, a syringe containing 20 intrarectal infectious doses in two ml of RPMI-1640 plus 10% fetal bovine serum (FBS) was attached to the tube and the inoculum slowly injected into the rectum. Following delivery of the inoculum, the feeding tube was flushed with 3.0 ml of RPMI without fetal calf serum and then slowly withdrawn. Animals were left in place, with pelvic regions slightly elevated, for a period of ten minutes following the challenge.

## Vaccine-Raised T-Cell Responses

DNA priming followed by rMVA boosting generated high frequencies of virus-specific T cells that peaked at one week following the rMVA booster, as shown in FIG. 15. The frequencies of T cells recognizing the Gag-CM9 epitope were assessed using Mamu-A\*01-tetramers; and the frequencies of T cells recognizing epitopes throughout Gag and Env, using pools of overlapping Gag and Env peptides and an enzyme linked immunospot (ELISPOT) assay.

For tetramer analyses, approximately  $1 \times 10^6$  PBMC were surface stained with antibodies to CD3 (FN-18, Biosource International, Camarillo, Calif.), CD8 (SK1, Becton Dickinson, San Jose, Calif.), and Gag-CM9 (CTPYDINQM)-Mamu-A\*01 tetramer conjugated to FITC, PerCP and APC respectively, in a volume of 100  $\mu$ l at 8-10° C. for 30 min. Cells were washed twice with cold PBS containing 2% FBS, fixed with 1% paraformaldehyde in PBS and analyses acquired within 24 hrs. on a FACScaliber (Becton Dickinson, San Jose, Calif.). Cells were initially gated on lymphocyte populations using forward scatter and side scatter and then on CD3 cells. The CD3 cells were then analyzed for CD8 and tetramer-binding cells. Approximately 150,000 lymphocytes were acquired for each sample. Data were analyzed using FloJo software (Tree Star, Inc. San Carlos, Calif.).

For IFN- $\gamma$  ELISPOTs, MULTISCREEN 96 well filtration plates (Millipore Inc. Bedford, Mass.) were coated overnight with anti-human IFN- $\gamma$  antibody (Clone B27, Pharmingen, San Diego, Calif.) at a concentration of 2  $\mu$ g/ml in sodium bicarbonate buffer (pH 9.6) at 8-10° C. Plates were washed two times with RPMI medium then blocked for one hour with complete medium (RPMI containing 10% FBS) at 37° C. Plates were washed five more times with plain RPMI medium and cells were seeded in duplicate in 100  $\mu$ l complete medium at numbers ranging from  $2 \times 10^4$  to  $5 \times 10^5$  cells per well. Peptide pools were added to each well to a final concentration of 2  $\mu$ g/ml of each peptide in a volume of 100  $\mu$ l in complete medium. Cells were cultured at 37° C. for about 36 hrs under 5% CO<sub>2</sub>. Plates were washed six times with wash buffer (PBS with 0.05% Tween-20) and then incubated with 1  $\mu$ g of biotinylated anti-human IFN- $\gamma$  antibody per ml (clone 7-86-1, Diapharma Group Inc., West Chester, Ohio) diluted in wash buffer containing 2% FBS. Plates were incubated for 2 hrs at 37° C. and washed six times with wash buffer. Avidin-HRP (Vector Laboratories Inc, Burlingame, Calif.) was added to each well and incubated for 30-60 min at 37° C. Plates were washed six times with wash buffer and spots were developed using stable DAB as substrate (Research Genetics Inc., Huntsville, Ala.). Spots were counted using a stereo dissecting microscope. An ovalbumin peptide (SIINFEKL) was included as a control in each analysis. Background spots for the ovalbumin peptide were generally <5 for  $5 \times 10^5$  PBMC s. This background when normalized for  $1 \times 10^6$  PBMC is <10. Only ELISPOT counts of twice the background ( $\geq 20$ ) were considered significant. The frequencies of ELISPOTs are approximate because different dilutions of cells have different efficiencies of spot formation in the absence of feeder cells (34). The same dilution of cells was used for all animals at a given time point, but different dilutions were used to detect memory and peak effector responses.

Simple linear regression was used to estimate correlations between post-booster and post-challenge ELISPOT responses, between memory and post-challenge ELISPOT responses, and between log viral loads and ELISPOT frequencies in vaccinated groups. Comparisons between vac-

cine and control groups were performed by means of 2-sample t-tests using log viral load and log ELISPOT responses. Comparisons of ELISPOTs or log viral loads between A\*01 and non A\*01 macaques were done using 2-sample t-tests. Two-way analyses of variance were used to examine the effects of dose and route of administration on peak DNA/MVA ELISPOTs, memory DNA/MVA ELISPOTs, and on logarithmically transformed Gag antibody data.

Gag-CM9 tetramer analyses were restricted to macaques that expressed the Mamu-A\*01 histocompatibility type, whereas ELISPOT responses did not depend on a specific histocompatibility type. Temporal T cell assays were designed to score both the acute (peak of effector cells) and long-term (memory) phases of the T cell response (FIG. 15A). As expected, the DNA immunizations raised low levels of memory cells that expanded to high frequencies within one week of the rMVA booster (FIG. 15). In Mamu-A\*01 macaques, cells specific to the Gag-CM9 epitope expanded to frequencies as high as 19% of total CD8 T cells (see animal 2 FIG. 15B). This peak of specific cells underwent a >10-fold contraction into the DNA/MVA memory pool (FIGS. 15A and B). ELISPOTs for three pools of Gag peptides also underwent a major expansion (frequencies up to 4000 spots for  $1 \times 10^6$  PBMC) before contracting into the DNA/MVA memory response (FIG. 15C). The frequencies of ELISPOTs were the same in macaques with and without the A\*01 histocompatibility type ( $P > 0.2$ ). At both peak and memory phases of the vaccine response, the rank order for the height of the ELISPOTs in the different vaccine groups was 2.5 mg i.d. > 2.5 mg i.m. > 250  $\mu$ g i.d. > 250  $\mu$ g i.m. (FIG. 15C). The IFN- $\gamma$ -ELISPOTs included both CD4 and CD8 cells (work in progress). Gag-CM9-specific CD8 cells had good lytic activity following restimulation with peptide (data not shown).

Impressively, in the outbred population of animals, pools of peptides throughout Gag and Env stimulated IFN- $\gamma$ -ELISPOTs (FIG. 16A). The breadth of the cellular response was tested at 25 weeks after the rMVA boost, a time when vaccine-raised T cells were in memory. Seven out of 7 pools of Gag peptides and 16 out of 21 pools of Env peptides were recognized by T cells in vaccinated animals. Of the five Env pools that were not recognized, two have been recognized in a macaque DNA/MVA vaccine trial at the U.S. Centers for Disease Control (data not shown). The remaining three (pools 19-21) had been truncated in our immunogens (Amara et al, 2001, submitted) and served as negative controls. Gag and Env ELISPOTs had overall similar frequencies in the DNA/MVA memory response (FIG. 16B). The greatest breadth of response was in high-dose i.d. DNA-primed animals where on average 10 peptide pools (4.5 Gag and 5.3 Env) were recognized. The rank order of the vaccine groups for breadth was the same as for the peak DNA/MVA response: 2.5 mg i.d. > 2.5 mg i.m. > 250  $\mu$ g i.d. > 250  $\mu$ g i.m. (FIG. 16B).

## EXAMPLE 15

## Challenge and Protection Against Aids

The highly pathogenic SHIV-89.6P challenge was administered intrarectally at 7 months after the rMVA booster, when vaccine-raised T cells were in memory (FIG. 15).

Determination of SHIV copy number: viral RNA from 150  $\mu$ l of ACD anticoagulated plasma was directly extracted with the QIAamp Viral RNA kit (Qiagen), eluted in 60  $\mu$ l AVE buffer, and frozen at -80° C. until SHIV RNA quantitation was performed. 5  $\mu$ l of purified plasma RNA was reverse transcribed in a final 20  $\mu$ l volume containing 50 mM KCl, 10

31

mM Tris-HCl, pH 8.3, 4 mM MgCl<sub>2</sub>, 1 mM each dNTP, 2.5 μM random hexamers, 20 units MultiScribe RT, and 8 units RNase inhibitor. Reactions were incubated at 25° C. for 10 min., followed by incubation at 42° C. for 20 min. and inactivation of reverse transcriptase at 99° C. for 5 min. The reaction mix was adjusted to a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 4 mM MgCl<sub>2</sub>, 0.4 mM each dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 0.1 μM probe and 5 units AmpliTaq Gold DNA polymerase (all reagents from Perkin Elmer Applied Biosystems, Foster City, Calif.). The primer sequences within a conserved portion of the SIV gag gene are the same as those described previously (Staprans, S., et al., 1996).

A Perkin Elmer Applied Biosystems 7700 Sequence Detection System was used with the PCR profile: 95° C. for 10 min., followed by 40 cycles at 93° C. for 30 sec., 59.5° C. for 1 min. PCR product accumulation was monitored using the 7700 sequence detector and a probe to an internal conserved gag gene sequence, where FAM and Tamra denote the reporter and quencher dyes. SHIV RNA copy number was determined by comparison to an external standard curve consisting of virion-derived SIVmac239 RNA quantified by the SIV bDNA method (Bayer Diagnostics, Emeryville, Calif.). All specimens were extracted and amplified in duplicate, with the mean result reported. With a 0.15-ml plasma input, the assay has a sensitivity of 10<sup>3</sup> copies RNA/ml plasma, and a linear dynamic range of 10<sup>3</sup> to 10<sup>8</sup> RNA copies (R<sup>2</sup>=0.995). The intra-assay coefficient of variation is <20% for samples containing >10<sup>4</sup> SHIV RNA copies/ml, and <25% for samples containing 10<sup>3</sup>-10<sup>4</sup> SHIV RNA copies/ml. In order to more accurately quantitate low SHIV RNA copy number in vaccinated animals at weeks 16 and 20, the following modifications to increase the sensitivity of the SHIV RNA assay were made: 1) Virions from ≤1 ml of plasma were concentrated by centrifugation at 23,000 g, 10° C. for 150 minutes and viral RNA was extracted; 2) A one-step RT-PCR method was used. Absolute SHIV RNA copy numbers were determined by comparison to the same SIVmac239 standards. These changes provided a reliable quantitation limit of 300 SHIV RNA copies/ml, and gave SHIV RNA values that were highly correlated to those obtained by the first method used (r=0.91, p<0.0001).

Challenge results: The challenge infected all of the vaccinated and control animals. However, by two weeks post-challenge, titers of plasma viral RNA were at least 10-fold lower in the vaccine groups (geometric means of 1×10<sup>7</sup> to 5×10<sup>7</sup>) than in the control animals (geometric mean of 4×10<sup>8</sup>) (FIG. 19A). By 8 weeks post-challenge, both high-dose DNA-primed groups and the low-dose i.d. DNA-primed group had reduced their geometric mean loads to about 1000 copies of viral RNA per ml. At this time the low-dose i.m. DNA-primed group had a geometric mean of 6×10<sup>3</sup> copies of viral RNA and the non-vaccinated controls, a geometric mean of 2×10<sup>6</sup>. By 20 weeks post-challenge, even the low-dose i.m. group had reduced its geometric mean copies of viral RNA to 1000. At this time, the unvaccinated controls were succumbing to AIDS. Among the 24 vaccinated animals, only one animal, in the low dose i.m. group, had intermittent viral loads above 1×10<sup>4</sup> copies per ml (FIG. 19D).

The rapid reduction of viral loads protected the vaccinated macaques against the loss of CD4 cells and the rapid onset of AIDS (FIGS. 19B, 19C, 19E). By 5 weeks post-challenge, all of the non-vaccinated controls had undergone the profound depletion of CD4 cells that is characteristic of SHIV-89.6P infections (FIG. 19B). All of the vaccinated animals maintained their CD4 cells with the exception of animal 22 (see above), which underwent a slow CD4 decline (FIG. 19E). By

32

23 weeks post-challenge, three of the four control animals had succumbed to AIDS (FIG. 19C). These animals had variable degrees of enterocolitis with diarrhea, cryptosporidiosis, colicystitis, enteric campylobacter infection, splenomegaly, lymphadenopathy, and SIV-associated giant cell pneumonia. In contrast, all 24 vaccinated animals have maintained their health.

Intracellular cytokine assays: Approximately 1×10<sup>6</sup> PBMC; were stimulated for one hour at 37° C. in 5 ml polypropylene tubes with 100 μg of Gag-CM9 peptide (CT-PYDINQM; SEQ ID NO:44) per ml in a volume of 100 μl RPMI containing 0.1% BSA and anti-human CD28 and anti-human CD49d (Pharmingen, Inc. San Diego, Calif.) costimulatory antibodies (1 μg/ml). 900 RPMI containing 10% FBS and monensin (10 μg/ml) was added and the cells cultured for an additional 5 hrs at 37° C. at an angle of 5 degrees under 5% CO<sub>2</sub>. Cells were surface stained with antibodies to CD8 conjugated to PerCP (clone SK1, Becton Dickinson) at 8°-10° C. for 30 min., washed twice with cold PBS containing 2% FBS, fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen, Inc.). Cells were then incubated with antibodies to human CD3 (clone FN-18, Biosource International, Camarillo, Calif.) and IFN-γ (Clone B27, Pharmingen) conjugated to FITC and PE, respectively, in Perm wash solution (Pharmingen) for 30 min at 4° C. Cells were washed twice with Perm wash, once with plain PBS, and resuspended in 1% para-formaldehyde in PBS. Approximately 150,000 lymphocytes were acquired on the FACScaliber and analyzed using FloJo software.

Proliferation assay: Approximately 0.2 million PBMC were stimulated with appropriate antigen in triplicate in a volume of 200 μl for five days in RPMI containing 10% FCS at 37° C. under 5% CO<sub>2</sub>. Supernatants from 293T cells transfected with the DNA expressing either SHIV-89.6 Gag and Pol or SHIV-89.6 Gag, Pol and Env were used directly as antigens. Supernatants from mock DNA (vector alone) transfected cells served as negative controls. On day six cells were pulsed with 1 μCi of tritiated-thymidine per well for 16-20 hrs. Cells were harvested using an automated cell harvester (TOMTEC, Harvester 96, Model 1010, Hamden, Conn.) and counted using a Wallac 1450 MICROBETA Scintillation counter (Gaithersburg, Md.). Stimulation indices are the counts of tritiated-thymidine incorporated in PBMC stimulated with 89.6 antigens divided by the counts of tritiated-thymidine incorporated by the same PBMC stimulated with mock antigen.

Post-challenge T cell results: Containment of the viral challenge was associated with a burst of antiviral T cells (FIG. 15; FIG. 20A). At one-week post challenge, the frequency of tetramer+ cells in the peripheral blood had decreased, potentially reflecting the recruitment of specific T cells to the site of infection (FIG. 20A). However, by two weeks post-challenge, tetramer+ cells in the peripheral blood had expanded rapidly, to frequencies as high, or higher, than after the MVA booster (FIGS. 15, 20A). The majority of the tetramer+ cells produced IFN-γ in response to a 6-hour stimulation with peptide Gag-CM9 (FIG. 20B) and did not have the "stunned" IFN-γ negative phenotype sometimes observed in chronic viral infections. The post-challenge burst of T cells contracted concomitant with the decline of the viral load. By 12 weeks post-challenge, virus-specific T cells were present at approximately one tenth of their peak height (FIGS. 15A, 20A, and data not shown). The height of the peak DNA/MVA-induced ELISPOTs presaged the height of the post-challenge T cell response as measured by ELISPOTs (r=+0.79, P<0.0001). In contrast to the vigorous secondary response in the vaccinated animals, the naive animals mounted a modest primary

response (FIGS. 15B, 15C and 20A). Tetramer+ cells peaked at less than 1% of total CD8 cells (FIG. 20A), and IFN- $\gamma$ -producing T cells were present at a mean frequency of about 300 as opposed to the much higher frequencies of 1000 to 6000 in the vaccine groups (FIG. 15C) ( $P < 0.05$ ). The tetramer+ cells in the control group, like those in the vaccine group, were largely IFN- $\gamma$  producing following stimulation with the Gag-CM9 peptide (FIG. 20B). By 12 weeks post challenge, 3 of the 4 controls had undetectable levels of IFN- $\gamma$ -producing T cells (data not shown). This rapid loss of anti-viral CD8 cells in the presence of high viral loads may reflect the lack of CD4 help.

T cell proliferative responses demonstrated that virus-specific CD4 cells had survived the challenge and were available to support the antiviral immune response (FIG. 20C). At 12 weeks post-challenge, mean stimulation indices for Gag-Pol-Env or Gag-Pol proteins ranged from 35 to 14 in the vaccine groups but were undetectable in the control group. Consistent with the proliferation assays, intracellular cytokine assays demonstrated the presence of virus-specific CD4 cells in vaccinated but not control animals (data not shown). The overall rank order of the vaccine groups for the magnitude of the proliferative response was 2.5 mg i.d. > 2.5 mg i.m. > 250  $\mu$ g i.d. > 250  $\mu$ g i.m.

Preservation of lymph nodes: At 12 weeks post-challenge, lymph nodes from the vaccinated animals were morphologically intact and responding to the infection whereas those from the infected controls had been functionally destroyed (FIG. 5). Nodes from vaccinated animals contained large numbers of reactive secondary follicles with expanded germinal centers and discrete dark and light zones (FIG. 5A). By contrast, lymph nodes from the non-vaccinated control animals showed follicular and paracortical depletion (FIG. 5B), while those from unvaccinated and unchallenged animals displayed normal numbers of minimally reactive germinal centers (FIG. 5C). Germinal centers occupied <0.05% of total lymph node area in the infected controls, 2% of the lymph node area in the uninfected controls, and up to 18% of the lymph node area in the vaccinated groups (FIG. 5D). The lymph node area occupied by germinal centers was about two times greater for animals receiving low-dose DNA priming than for those receiving high-dose DNA priming, suggesting more vigorous immune reactivity in the low-dose animals (FIG. 5D). At 12 weeks post-challenge, in situ hybridization for viral RNA revealed rare virus-expressing cells in lymph nodes from 3 of the 24 vaccinated macaques, whereas virus-expressing cells were readily detected in lymph nodes from each of the infected control animals (FIG. 5E). In the controls, which had undergone a profound depletion in CD4 T cells, the cytomorphology of infected lymph node cells was consistent with a macrophage phenotype (data not shown).

Temporal antibody response: ELISAs for total anti-Gag antibody used bacterial produced SIV gag p27 to coat wells (2  $\mu$ g per ml in bicarbonate buffer). ELISAs for anti-Env antibody used 89.6 Env produced in transiently transfected 293T cells captured with sheep antibody against Env (catalog number 6205; International Enzymes, Fairbrook Calif.). Standard curves for Gag and Env ELISAs were produced using serum from a SHIV-89.6-infected macaque with known amounts of anti-Gag or anti-Env IgG. Bound antibody was detected using goat anti-macaque IgG-PO (catalog # YNGMOIGGFPC, Accurate Chemical, Westbury, N.Y.) and TMB substrate (Catalog # T3405, Sigma, St. Louis, Mo.). Sera were assayed at 3-fold dilutions in duplicate wells. Dilutions of test sera were performed in whey buffer (4% whey and 0.1% tween 20 in 1xPBS). Blocking buffer consisted of whey buffer plus 0.5% non-fat dry milk. Reactions were stopped with 2M

H<sub>2</sub>SO<sub>4</sub> and the optical density read at 450 nm. Standard curves were fitted and sample concentrations were interpolated as  $\mu$ g of antibody per ml of serum using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, Calif.).

Results showed that the prime/boost strategy raised low levels of anti-Gag antibody and undetectable levels of anti-Env antibody (FIG. 22). However, post-challenge, antibodies to both Env and Gag underwent anamnestic responses with total Gag antibody reaching heights approaching one mg per ml and total Env antibody reaching heights of up to 100  $\mu$ g per ml (FIGS. 22A and B).

By two weeks post-challenge, neutralizing antibodies for the 89.6 immunogen, but not the SHIV-89.6P challenge were present in the high-dose DNA-primed groups (geometric mean titers of 352 in the i.d. and 303 in the i.m. groups) (FIG. 22C). By 5 weeks post-challenge, neutralizing antibody to 89.6P had been generated (geometric mean titers of 200 in the high-dose i.d. and 126 in the high-dose i.m. group) (FIG. 22D) and neutralizing antibody to 89.6 had started to decline. Thus, priming of an antibody response to 89.6 did not prevent a B cell response leading to neutralizing antibody for SHIV-89.6P. By 16 to 20 weeks post-challenge, antibodies to Gag and Env had fallen in most animals (FIGS. 22A and B). This would be consistent with the control of the virus infection.

T cells correlate with protection. The levels of plasma viral RNA at both two and three weeks post-challenge correlated inversely with the peak pre-challenge frequencies of DNA/MVA-raised IFN- $\gamma$  ELISPOTs ( $r = -0.53$ ,  $P = 0.008$  and  $r = -0.70$ ,  $P = 0.0002$  respectively) (FIG. 23A).

Importantly, these correlations were observed during the time the immune response was actively reducing the levels of viremia. At later times post-challenge, the clustering of viral loads at or below the level of detection precluded correlations. Correlations also were sought between viral load and post-challenge ELISPOT, proliferative, and neutralizing antibody responses. The levels of IFN- $\gamma$  ELISPOTS at two weeks post-challenge correlated with the viral load at 3 weeks post-challenge ( $r = -0.51$ ,  $P = 0.009$ ) (data not shown). Post-challenge proliferative and neutralizing antibody responses did not correlate with viral loads.

Dose and route: The dose of DNA had significant effects on both cellular and humoral responses ( $P < 0.05$ ) while the route of DNA administration had a significant effect only on humoral responses (FIGS. 23 C-E). The intradermal route of DNA delivery was about 10 times more effective than the intramuscular route for generating antibody to Gag ( $P = 0.02$ ) (FIG. 23E). Within our data set, i.d. DNA injections were about 3 times more effective at priming the height and breadth of virus-specific T cells (FIGS. 23C and D). However, these differences were not significant (height,  $P = 0.2$ ; breadth,  $P = 0.08$ ). Interestingly, the route and dose of DNA had no significant effect on the level of protection. At 20 weeks post-challenge, the high-dose DNA-primed animals had slightly lower geometric mean levels of viral RNA ( $7 \times 10^2$  and  $5 \times 10^2$ ) than the low-dose DNA-primed animals ( $9 \times 10^2$  and  $1 \times 10^3$ ). The animal with the highest intermittent viral loads (macaque 22) was in the low dose i.m.-primed group (FIG. 19D). Thus, the low dose i.m.-primed group, which was slow to control viremia (FIG. 19A), may have poorer long term protection. The breadth of the response did not have an immediate effect on the containment of viral loads, but with time may affect the frequency of viral escape.

These results clearly demonstrate that a multiprotein DNA/MVA vaccine can raise a memory immune response capable of controlling a highly virulent mucosal immunodeficiency virus challenge. Our excellent levels of viral control are more favorable than have been achieved using only DNA or rMVA



## 35

vaccines (Egan et al., 2000; I. Ourmanov et al., 2000) and comparable to those obtained for DNA immunizations adjuvanted with interleukin-2 (Barouch et al., 2000). All of these previous studies have used more than three vaccine inoculations, none have used mucosal challenges, and most have challenged at peak effector responses and not allowed a prolonged post vaccination period to test for "long term" efficacy as was done in our study. Our results also demonstrate for the first time that vaccine-raised T cells, as measured by IFN- $\gamma$  ELISPOTs, are a correlate for the control of viremia. This relatively simple assay can now be used for preclinical evaluation of DNA and MVA immunogens for HIV-1, and should be able to be used as a marker for the efficacy of clinical trials in humans.

The DNA/MVA vaccine did not prevent infection. Rather, the vaccine controlled the infection, rapidly reducing viral loads to near or below 1000 copies of viral RNA per ml of blood. Containment, rather than prevention of infection, affords the virus the opportunity to establish a chronic infection (Chun et al., 1998). Nevertheless, by rapidly reducing viral loads, a multiprotein DNA/MVA vaccine will extend the prospect for long-term non-progression and limit HIV transmission.

## EXAMPLE 16

## Gag-Pol Vaccine Trial

A trial using Gag-Pol rather than Gag-Pol-Env expressing immunogens was conducted to determine the importance of including Env in the vaccine (see FIG. 27 for constructs). A vaccine that did not include Env would have certain advantages in the field, such as the ability to screen for anti-Env antibody as a marker for infection. This trial used pGA1/Gag-Pol and a rMVA expressing the Gag-Pol sequences of SIV239 (MVA/Gag-Pol) supplied by Dr. Bernard Moss (NIH-NIAID)

The "Gag-Pol" immunogens were administered using the schedule described in Example 13 above for the "Gag-Pol-Env" (pGA2/89.6 MVA/89.6) immunogens (see Table 4, Groups 5 and 6). The same doses of DNA, 2.5 mg and 250  $\mu$ g, were used to prime a high dose and a low dose group and administration was via an intradermal route. As in the previous vaccine trial described in examples 13-15, two to three mamu A\*01 macaques were included in each trial group. T cell responses were followed for those specific for the p11c-m epitope using the p11c-m tetramers and using ELISPOTs stimulated by pools of overlapping peptides, as described in the above Examples.

Following immunization, vaccine recipients showed anti-Gag T cell responses similar to those observed in the Gag-Pol-Env vaccine trial. Animals were challenged intrarectally with SHIV-89.6P at 7.5 months following the rMVA booster (FIG. 28). In contrast to the Gag-Pol-Env vaccine protocol, which protected animals against the rapid loss of CD4 cells, the Gag-Pol animals uniformly lost CD4 cells (FIGS. 28B and 28D). This loss was most pronounced in the group receiving the low dose i.d. DNA prime. Consistent with the loss of CD4 cells, the Gag-Pol DNA-immunized groups were also less effective at reducing their viral loads than the Gag-Pol-Env groups (FIGS. 28A and 28C). Geometric mean viral loads for these groups were 10-100-fold higher at 3 weeks post challenge and 10 fold higher at 5 weeks post challenge. These results demonstrate that the Env gene plays an important role in protecting CD4 cells and reducing the levels of viral RNA in challenged animals. The results also show that

## 36

Gag-Pol-Env DNA/MVA vaccines function more effectively than Gag-Pol DNA/MVA vaccines in protecting recipients against a virulent challenge.

## EXAMPLE 17

## Measles Inserts

A DNA vaccine expressing a fusion of measles H and the C3d component of complement was used to determine if vaccination could achieve earlier and more efficient anti-H antibody responses. In prior studies in mice by Dempsey et al., the fusion of two or three copies of C3d to a model antigen, hen egg lysozyme increased the efficiency of immunizations by more than 1000-fold (Dempsey et al, 1996). This resulted in more rapid appearance of hemagglutination inhibition (HI) activity and protective immunity (Ross et al, 2000 and Ross et al., 2001).

In the human immune system, one consequence of complement activation is the covalent attachment of the C3d fragment of the third complement protein to the activating protein. C3d in turn binds to CD21 on B lymphocytes, a molecule with B cell stimulatory functions that amplify B lymphocyte activation. In a measles H-C3d fusion protein, the H moiety of the fusion would bind to anti-H Ig receptors on B cells and signal through the B cell receptor, while the C3d moiety of the fusion would bind to CD21 and signal through CD19. In this hypothesis, a B cell responding to an H-C3d fusion protein would undergo more effective signaling than a B cell responding to H alone. Mice vaccinated with DNA expressing a secreted H-fused to three copies of C3d (sH-3C3d) generated a more rapid appearance and higher levels of neutralizing antibody activity than DNA expressing sH only.

Plasmid DNA: pTR600, a eukaryotic expression vector, was constructed to contain two copies of the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains a multicloning site for the easy insertion of gene segments and the Col E1 origin of replication for prokaryotic replication and the Kanamycin resistance gene (Kan<sup>r</sup>) for selection in antibiotic media (FIG. 29A).

Hemagglutinin (H) cDNA sequences from the Edmonton strain and C3d sequences were cloned as previously described and transferred into the pTR600 vaccine vector using unique restriction endonuclease sites (FIG. 29B). The secreted version was generated by deleting the transmembrane and cytoplasmic domains of H. This was accomplished using PCR to clone a fragment of the H gene in frame with an N-terminal synthetic mimic of the tissue plasminogen activator (tpA) leader sequence (Torres, et al, 2000).

The vectors expressing sH-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homologue of C3d in frame at the 3' end of the sH gene as previously described (Dempsey, 1996; Ross et al, 2000; and Ross et al, 2001). The construct design was based upon Dempsey et al. and used sequences from pSLG-C3d. Linkers composed of two repeats of 4 glycines and a serine  $\{(G_4S)_2\}$  were fused at the junctures of H and C3d and between each C3d repeat. Potential proteolytic cleavage sites between the junctions of C3d and the junction of sH and C3d were mutated by using Bam HI and Bgl II fusion to mutate an Arg codon to a Gly codon.

The plasmids were amplified in *Escherichia coli* strain, DH5 $\alpha$ , purified using anion-exchange resin columns (Qiagen, Valencia, Calif.) and stored at -20° C. in dH<sub>2</sub>O.

Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260 nm and 280 nm.

Mice and DNA immunizations: Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were used for inoculations. Briefly, mice were anesthetized with 0.03-0.04 ml of a mixture of 5 ml ketamine HCl (100 mg/ml) and 1 ml xylazine (20 mg/ml). Mice were immunized with two gene gun doses containing 0.5 µg of DNA per 0.5 mg of approximately 1-µm gold beads (DeGussa-Huls Corp., Ridgefield Park, N.J.) at a helium pressure setting of 400 psi.

Transfections and expression analysis: The human embryonic kidney cell line 293T ( $5 \times 10^5$  cells/transfection) was transfected with 2 µg of DNA using 12% lipofectamine according to the manufacture's guidelines (Life Technologies, Grand Island, N.Y.). Supernatants were collected and stored at -20° C. Quantitative antigen capture ELISAs for H were conducted as previously described (Cardoso et al, 1998).

For western hybridization analysis, 15 µl of supernatant or cell lysate was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, Calif.) and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, Calif.) and incubated with a 1:1000 dilution of polyclonal rabbit anti-HA antisera in PBS containing 0.1% Tween 20 and 1% nonfat dry milk. After extensive washing, bound rabbit antibodies were detected using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Antibody assays: A quantitative ELISA was performed to assess anti-H specific IgG levels. Briefly, Ltk<sup>-</sup> cells constitutively expressing the H protein of MV (24) were grown in 96-well plates. Antisera dilutions were incubated with the intact cells expressing H antigen. The anti-H antibodies were allowed to bind to the cells for 30 min following which the cells were fixed in acetone (80%). The specific antibody responses were detected with biotinylated anti-mouse IgG antibodies and the streptavidine-phosphatase alkaline system (Sigma). Antibody binding to Ltk<sup>-</sup> cells not expressing H antigen was used to standardize the system. The results were expressed as the endpoint dilution titer.

Neutralization assays. Neutralization assays were conducted on Vero cells grown in six well plates (25). Briefly, 100-200 p.f.u. of the Edmonton strain of measles virus were mixed with serial dilution of sera, incubated for 1 h at 37° C. and then inoculated onto plates. Plates were incubated at 37° C. for 48 h and plaques were counted. Neutralization titers are defined as the reciprocal dilution of sera required to reduce plaque formation by 50% or 90%. Preimmune sera served as negative controls.

Results: Two hemagglutinin-expressing vaccine plasmids were constructed in the pTR600 vector to express either a secreted form of H (sH) from the Edmonston strain or a C3d-fusion of the secreted form of H (sH-3C3d) (FIG. 29). The sH represented the entire ectodomain of H, but excluded the transmembrane and cytoplasmic region. The cloning placed the N-terminal synthetic mimic of the tissue plasminogen activator (tPA) leader sequence in frame with the H sequence. The tPA leader and H sequences were fused immediately 3' to the transmembrane domain of H. The sH-3C3d fusion protein was generated by cloning three tandem repeats of the mouse homologue of C3d in frame with the secreted H gene (FIG. 29B). The proteolytic cleavage sites, found at the

junction between each C3d molecule as well as the junction between the H protein and the first C3d coding region, were destroyed by mutagenesis.

Western blot analyses revealed sH and sH-3C3d proteins of the expected sizes. Using a rabbit polyclonal antibody to MV H antisera, western blot analysis showed a broad band of ~70 kD corresponding to the secreted form of H in the supernatant of transfected cells. A higher molecular weight band at ~190 kD is consistent with the projected size of the sH-3C3d fusion protein (FIG. 30). No evidence for the proteolytic cleavage of the sH-C3d fusion protein was seen by western analysis.

Measles virus H was expressed at slightly lower levels by plasmids containing either the sH or sH-3C3d compared to transmembrane-associated forms of the antigen. Human 293T cells were transiently transfected with 2 µg of plasmid and both supernatants and cell lysates were assayed for H using an antigen capture ELISA. Approximately 75% of the H protein was secreted into the supernatant for both sH-DNA and sH-3C3d-DNA transfected cells. As expected, ~99% of the H antigen was detected in the cell lysate of cells transfected with plasmids expressing transmembrane form of H.

Antibody Response to Measles H DNA Immunizations: The sH-3C3d expressing DNA plasmids raised higher titers of ELISA antibody than sH DNA. BALB/c mice were vaccinated by DNA coated gold particles via gene gun with either a 0.1 µg or a 1 µg inoculum. At 4 and 26 weeks post vaccination, mice were boosted with the same dose of DNA given in the first immunization. The temporal pattern for the appearance of anti-H antibody showed a faster onset in mice vaccinated with the C3d fusion expressing DNA compared to mice vaccinated with sH DNA. Good titers of antibody were raised by the first immunization. These were boosted by the 2<sup>nd</sup> and 3<sup>rd</sup> immunizations following the third immunization, titers were 5-6 times higher in the sH-3C3d vaccinated mice than in those vaccinated with sH DNA.

Neutralization assays: Examination of the serum for MV neutralization showed titers up to 1700 after the second inoculation of 0.1 µg of sH-3C3d expressing DNA. Neutralizing antibody studies performed on Vero cells detected higher titers of neutralizing activity against the prototype MV Edmonton strain in mouse sera elicited by the sH-3C3d constructs than in the sera of mice vaccinated with sH expressing DNA. Mice vaccinated with sH-3C3d expressing plasmids had a sharp rise in neutralizing antibody levels that reached a plateau by week 14. In contrast, it took a third vaccination with sH expressing DNA to elicit detectable levels of neutralizing antibodies. After 28 weeks post-vaccination, sera from mice vaccinated with sH-3C3d-DNA had neutralizing titers (>250) that could reduce plaque formation of MV infection by 90%.

The increase in height of the antibody response to H was 7-15 fold higher in mice vaccinated with the C3d protein expressing constructs compared to mice vaccinated with DNA expressing sH only. The increase in antibody response with DNA expressing sH-3C3d is even more intriguing, since this plasmid expressed ~60% as much protein as plasmid expressing sH only.

In addition to the increase in the overall antibody level, there was a faster onset of antibodies that could specifically neutralize MV in an in vitro infection assay. After the second immunization, detectable levels of neutralizing antibodies were observed in mice vaccinated with DNA expressing sH-3C3d. The titer of the neutralizing antibody peaked at week 14 (1700 for 50% plaque reduction), which are substantially above the minimum correlate for protection (>120 for 50% plaque reduction). In contrast, mice vaccinated with sH

expressing DNA had low levels of neutralizing antibody even after the third vaccination (180 for 50% plaque reduction) (FIG. 31).

#### EXAMPLE 18

##### Influenza Inserts with and without -C3d

Plasmid vector construction and purification procedures have been previously described for JW4303 (Torres, et al. 1999; Pertmer et al. 1995; Feltquate et al. 1997). In brief, influenza hemagglutinin (HA) sequences from A/PR/8/34 (H1N1) were cloned into either the pJW4303 or pGA eukaryotic expression vector using unique restriction sites.

Two versions of HA, a secreted(s) and a transmembrane (tm) associated, have been previously described (Torres et al. 1999; Feltquate et al., 1997). Vectors expressing sHA or tmHA in pJW4303 were designated pJW/sHA and pJW/tmHA respectively and the vectors expressing sHA, tmHA, or sHA-3C3d in pGA were designated pGA/sHA, pGA/tmHA, and pGA/sHA-3C3d respectively.

Vectors expressing HA-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homolog of C3d and placing the three tandem repeats in-frame with the secreted HA gene. The construct designed was based upon Dempsey et al. (1996). Linkers composed of two repeats of 4 glycines and a serine [(G<sub>4</sub>S)<sub>2</sub>] were fused at the joints of each C3d repeat. The pGA/sHA-3C3d plasmid expressed approximately 50% of the protein expressed by the pGA/sHA vector. However, the ratio of sHA-3C3d found in the supernatant vs. the cell lysate was similar to the ratio of antigen expressed by pGA/sHA. More than 80% of the protein was secreted into the supernatant. In western analysis, a higher molecular weight band was detected at 120 kDa and represented the sHA-3C3d fusion protein. Therefore, the sHA-3C3d fusion protein is secreted into the supernatant as efficiently as the sHA antigen.

Mice and DNA immunizations. Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were used for inoculations. Mice, housed in microisolator units and allowed free access to food and water, were cared for under USDA guidelines for laboratory animals. Mice were anesthetized with 0.03-0.04 ml of a mixture of 5 ml ketamine HCl (100 mg/ml) and 1 ml xylazine (20 mg/ml). Gene gun immunizations were performed on shaved abdominal skin using the hand held Accell gene delivery system and immunized with two gene gun doses containing 0.5 µg of DNA per 0.5 mg of approximately 1-µm gold beads (DeGussa-Huls Corp., Ridgefield Park, N.J.) at a helium pressure setting of 400 psi.

Influenza virus challenge. Challenge with live, mouse-adapted, influenza virus (A/PR/8/34) was performed by intranasal instillation of 50 µl allantoic fluid, diluted in PBS to contain 3 lethal doses of virus, into the nares of ketamine-anesthetized mice. This method leads to rapid lung infections and is lethal to 100% of non-immunized mice. Individual mice were challenge at either 8 or 14 weeks after vaccination and monitored for both weight loss and survival. Data were plotted as the average individual weight in a group, as a percentage of pre-challenge weight, versus days after challenge.

Antibody response to the HA DNA Immunization protocol: The tmHA and sHA-3C3d expressing DNA plasmids raised higher titers of ELISA antibody than the sHA DNA. BALB/c mice were vaccinated by DNA coated gold particles via gene gun with either a 0.1 µg or 1 µg dose inoculum. At 4 weeks post vaccination, half of the mice in each group were boosted with the same dose of DNA given in the first immu-

nization. Total anti-HA IgG induced by the sHA-3C3d- and tmHA-expressing plasmids were similar in the different experimental mouse groups and 3-5 times higher than the amount raised by the sHA expressing plasmids (FIG. 24). In addition, the amount of anti-HA antibody elicited increased relative to the amount of DNA used for vaccination in a dose dependent manner (FIG. 24E-24F). Overall, the dose response curves and temporal pattern for the appearance of anti-HA antibody were similar in the mice vaccinated with tmHA-DNA or sHA-3C3d-DNA, but lower and slower, in the mice vaccinated with sHA-DNA. As expected, the booster immunization both accelerated and increased the titers of antibodies to HA.

Avidity of mouse HA antiserum. Sodium thiocyanate (NaSCN) displacement ELISAs demonstrated that the avidity of the HA-specific antibody generated with sHA-3C3d expressing DNA was consistently higher than antibodies from sHA-DNA or tmHA-DNA vaccinated mice (FIG. 25). The avidity of specific antibodies to HA was compared by using graded concentrations NaSCN, a chaotropic agent, to disrupt antigen-antibody interactions. The binding of antibodies with less avidity to the antigen is disrupted at lower concentrations of NaSCN than that of antibodies with greater avidity to the antigen. The effective concentration of NaSCN required to release 50% of antiserum (ED<sub>50</sub>) collected at 8 weeks after vaccination from sHA-DNA or tmHA-DNA boosted mice (0.1 µg dose or 1 µg dose) was ~1.20 M (FIG. 25A). In contrast, antiserum from mice vaccinated and boosted with sHA-3C3d-DNA had an ED<sub>50</sub> of ~1.75 M (FIG. 25B). At the time of challenge (14 weeks after vaccination), the ED<sub>50</sub> had increased to ~1.8 M for antibodies from both sHA-DNA and tmHA-DNA vaccinated mice (FIG. 25C). Antibodies from mice vaccinated with sHA-3C3d-DNA had increased to an ED<sub>50</sub> of ~2.0 M (FIG. 25D). These results suggest that the antibody from sHA-3C3d-DNA vaccinated mice had undergone more rapid affinity maturation than antibody from either sHA-DNA or tmHA-DNA vaccinated mice. The difference between the temporal avidity maturation of antibody for sHA-3C3d and tmHA was independent of the level of the raised antibody. Both of these plasmids had similar temporal patterns for the appearance of antibody and dose response curves for the ability to raise antibody (FIG. 25).

Hemagglutinin-Inhibition (HI) titers. Hemagglutination-inhibition assays (HI) were performed to evaluate the ability of the raised antibody to block binding of A/PR/8/34 (H1N1) to sialic acid. The HI titers were measured from serum samples harvested from mice at 8 and 14 weeks after vaccination. All boosted mice had measurable HI titers at week 14 regardless of the dose or vaccine given. The highest titers (up to 1:1200) were recorded for the sHA-3C3d-DNA vaccinated mice. Nonboosted mice showed more variation in HI titers. Nonboosted mice vaccinated with a 0.1 µg dose of either sHA-DNA or tmHA-DNA expressing plasmids had low HI titers of 1:10. In contrast, mice vaccinated with sHA-3C3d-DNA had titers greater than 1:640. The only vaccinated mice that had a measurable HI titer (1:160) at week 8 were boosted mice vaccinated with 1 µg dose sHA-3C3d-DNA. These results indicate that C3d, when fused to sHA, is able to stimulate specific B cells to increase the avidity maturation of antibody and thus the production of neutralizing antibodies to HA.

Protective efficacy to influenza challenge. Consistent with eliciting the highest titers of HI antibody, the sHA-3C3dDNA raised more effective protection than the sHA or tmHA DNAs. To test the protective efficacy of the various HA-DNA vaccines, mice were challenged with a lethal dose of A/PR/8/34 influenza virus (H1N1) and monitored daily for morbid-

ity (as measured by weight loss) and mortality. Weight loss for each animal was plotted as a percentage of the average pre-challenge weight versus days after challenge (FIG. 26). Virus-challenged naive mice and pGA vector only vaccinated mice showed rapid weight loss with all the mice losing >20% of their body weight by 8 days post-challenge (FIG. 26). In contrast, PBS mock-challenged mice showed no weight loss over the 14 days of observation. All boosted mice survived challenge, 14 weeks after vaccination, regardless of the dose of DNA plasmid administered. However, boosted mice vaccinated with a 0.1 µg dose of sHA-DNA did drop to 92% of their initial body weight at 8 days post-challenge before recovering (FIG. 26). In contrast, when 1 µg dose, boosted mice were challenged at 8 weeks after vaccination, the only mice to survive challenge were sHA-3C3d- and tmHA-DNA vaccinated mice, albeit with greater weight loss than was observed from mice challenged at 14 weeks after vaccination. The only 0.1 µg dose, boosted mice to survive challenge at 8 weeks after vaccination were the sHA-3C3d vaccinated mice (FIG. 26).

Among the nonboosted, 0.1 µg dose immunizations, only the sHA-3C3d-DNA vaccinated mice survived challenge at 14 weeks after vaccination (FIG. 26). All mice administered a single DNA vaccination lost weight. However, of these, the sHA-3C3d-DNA vaccinated mice lost the least weight and these mice were the only mice to survive the lethal challenge (FIG. 26). These results demonstrate that 3C3d protein, when fused to HA, increased the efficiency of a DNA vaccine, allowing for the reduction in dose of DNA and the number of vaccinations needed to afford protection to a lethal influenza virus challenge.

#### EXAMPLE 19

##### HIV Gp120-C3d Fusion Constructs

In this study, a similar approach to that described in Example 18 was used to fuse three copies of murine C3d to the carboxyl terminus of HIV Env gp120 subunit. Using DNA vaccination, BALB/c mice were inoculated and assayed for enhanced immune responses. The fusion constructs induced higher antibody responses to Env and a faster onset of avidity maturation than did the respective wild-type gp120 sequences. These results suggest that the efficacy of DNA vaccines for raising antibody can be significantly improved by fusing proteins with C3d.

Plasmid DNA: pGA was constructed as described in Example 1 to contain the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts, and the bovine growth hormone polyadenylation signal (BGH polyA) for termination of transcription. HIV envelope sequences from the isolates ADA, IIIB and 89.6, encoding almost the entire gp120 region, and C3d sequences were cloned into the pGA vaccine vector using unique restriction endonuclease sites. The gp120 segment encoded a region from amino acid 32 to amino acid 465 and ended with the amino acid sequence VAPTRA (SEQ ID NO:45). The first 32 amino acids were deleted from the N-terminus of each sgp120 and replaced with a leader sequenced from the tissue plasminogen activator (tpA). The vectors expressing sgp120-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homologue of C3d in frame with the sgp120 expressing DNA. The construct design was based upon Dempsey et al. (1996). Linkers composed of two repeats of four glycine residues and a serine {G<sub>4</sub>S}<sub>2</sub> were fused at the junctures of HA and C3d and between each C3d repeat. Potential proteolytic cleavage sites

between the junctions of C3d and the junction of 3C3d were mutated by ligating Bam HI and Bgl II restriction endonuclease sites to mutate an Arg codon to a Gly codon.

The plasmids were amplified in *Escherichia coli* strain DH5α, purified using anion-exchange resin columns (Qiagen, Valencia, Calif.) and stored at -20° C. in dH<sub>2</sub>O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260 nm and 280 nm.

Mice and DNA immunizations: Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were vaccinated as described in Example 17 above. Briefly, mice were immunized with two gene gun doses containing 0.5 µg of DNA per 0.5 mg of approximately 1-µm gold beads (DeGussa-Huls Corp., Ridgefield Park, N.J.) at a helium pressure setting of 400 psi.

Transfections and expression analysis and western hybridization experiments were conducted as described in Example 17, except that the nitrocellulose membranes were incubated with a 1:1000 dilution of polyclonal human HIV-infected patient antisera in PBS containing 0.1% Tween 20 and 1% nonfat dry milk. After extensive washing, bound human antibodies were detected using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

ELISA and avidity assays: An endpoint ELISA was performed to assess the titers of anti-Env IgG in immune serum using purified HIV-1-IIIB gp120 CHO-expressed protein (Intracell) to coat plates as described (Richmond et al., 1998). Alternatively, plates were coated with sheep anti-Env antibody (International Enzymes Inc., Fallbrook, Calif.) and used to capture sgp120 produced in 293T cells that were transiently transfected with sgp120 expression vectors. Mouse sera from vaccinated mice was allowed to bind and subsequently detected by anti-mouse IgG conjugated to horseradish peroxidase. Endpoint titers were considered positive that were two fold higher than background. Avidity ELISAs were performed similarly to serum antibody determination ELISAs up to the addition of samples and standards. Samples were diluted to give similar concentrations of specific IgG by O.D. Plates were washed three times with 0.05% PBS-Tween 20. Different concentrations of the chaotropic agent, sodium thiocyanate (NaSCN) in PBS, were then added (0M, 1 M, 1.5 M, 2 M, 2.5 M, and 3 M NaSCN). Plates were allowed to stand at room temperature for 15 minutes and then washed six times With PBS-Tween 20. Subsequent steps were performed similarly to the serum antibody determination ELISA and percent of initial IgG calculated as a percent of the initial O.D. All assays were done in triplicate.

Neutralizing antibody assays: Antibody-mediated neutralization of HIV-1 IIIB and 89.6 was measured in an MT-2 cell-killing assay as described previously (Montefiori et al., 1988). Briefly, cell-free virus (50 µl containing 10<sup>8</sup> TCID<sub>50</sub> of virus) was added to multiple dilutions of serum samples in 100 µl of growth medium in triplicate wells of 96-well microtiter plates coated with poly-L-lysine and incubated at 37° C. for 1 h before MT-2 cells were added (10<sup>5</sup> cells in 100 µl added per well). Cell densities were reduced and the medium was replaced after 3 days of incubation when necessary. Neutralization was measured by staining viable cells with Finter's neutral red when cytopathic effects in control wells were >70% but less than 100%. Percentage protection was determined by calculating the difference in absorption (A<sub>540</sub>) between test wells (cells+virus) and dividing this result by the difference in absorption between cell control wells (cells

only) and virus control wells (virus only). Neutralizing titers are expressed as the reciprocal of the plasma dilution required to protect at least 50% of cells from virus-induced killing.

Results: Env was expressed at overall similar levels by plasmids containing either the secreted form of the antigen, but at a two-four-fold lower level by the sgp120-C3d expressing plasmids. Human 293T cells were transiently transfected with 2 µg of plasmid and both supernatants and cell lysates were assayed for gp120 using an antigen capture ELISA. The sgp120 constructs expressed from 450 to 800 ng per ml, whereas the 3C3d fusions expressed from 140 to 250 ng per ml. Approximately 90% of the Env protein was present in the supernatant for both sgp120 and sgp120-3C3d-DNA transfected cells (data not shown). The approximately 2-fold differences in the levels of expression of the different sgp120s is likely a reflection in differences in the Env genes as well as differences in the efficiency that the capture and detection antibodies recognized the different Envs.

Western blot analyses revealed sgp120 and sgp120-3C3d proteins of the expected sizes. Using human patient polyclonal antisera, western blot analysis showed the expected broad band of 115-120 kD corresponding to gp120. A higher molecular weight band at ~240 kD was consistent with the projected size of the sgp120-3C3d fusion protein. Consistent with the antigen-capture assay, intense protein bands were present in the supernatants of cells transfected with sgp120-DNA, whereas less intense bands were present in the supernatants of cells transfected with sgp120-3C3d-DNA (data not shown). No evidence for the proteolytic cleavage of the sgp120-C3d fusion protein was seen by western analysis.

Antibody response to Env gp120 DNA immunizations: The sgp120-3C3d expressing DNA plasmids raised higher titers of ELISA antibody than the sgp120 DNA. BALB/c mice were vaccinated by DNA coated gold particles via gene gun with a 1 µg dose inoculum. Mice were vaccinated at day 1 and then boosted at 4, 14, and 26 weeks with the same DNA given in the first immunization. When sera were assayed on gp120-IIIB-coated plates, mice vaccinated with the DNAs expressing the C3d fusion proteins had anti-Env antibodies 3-7 times higher than the amount of antibody raised by the counterpart sgp120 expressing plasmids. Among the C3d constructs, mice vaccinated with sgp120-(IIIB)-3C3d had the highest levels of antibody and mice vaccinated with sgp120-(ADA)-3C3d expressing DNA had the lowest levels of anti-Env antibodies. The temporal pattern for the appearance of anti-Env antibody revealed titers being boosted at each of the inoculations for all constructs tested.

Differences in the levels of the antibody raised by the different Envs appeared to be determined by the specificity of the raised antibody. Using an alternative ELISA protocol, in which antibody was captured on the homologous Env, all of the C3d-fusions appeared to raise similar levels of antibody. In this assay, sheep anti-Env antibody was used to capture transiently produced sgp120 proteins. This assay revealed low, but similar levels of antibody raised by each of the sgp120-3C3d constructs. The lower levels of antibody detected in this assay are likely to reflect the levels of transfection-produced Env used to capture antibody being lower than in the assays using commercially produced IIIB gp120 to coat plates. As expected using either ELISA method, booster immunizations were necessary to achieve even the most modest antibody response.

Avidity of mouse Env antiserum: Sodium thiocyanate (NaSCN) displacement ELISAs demonstrated that the avidity of the antibody generated with sgp120-3C3d expressing DNA was consistently higher than that from sgp120-DNA vaccinated mice. Avidity assays were conducted on sera

raised by sgp120-(IIIB) and sgp120-(IIIB)-3C3d because of the type specificity of the raised antisera and the commercial availability of the IIIB protein (but not the other proteins) for use as capture antigen. The avidity of specific antibodies to Env was compared by using graded concentrations NaSCN, a chaotropic agent, to disrupt antigen-antibody interaction. Results indicated that the antibody from sgp120-3C3d-DNA vaccinated mice underwent more rapid affinity maturation than antibody from sgp120-DNA vaccinated mice.

Env-3C3d expressing plasmids elicit modest neutralizing antibody: Neutralizing antibody studies performed on MT-2 cells detected higher titers of neutralizing activity in the sera generated by the gp120-3C3d constructs than in the sera generated by the sgp120 constructs. Sera were tested against two syncytium inducing, IIIB (X4) and 89.6 (X4R5) viruses. Mice vaccinated with sgp120-3C3d expressing plasmids had very modest levels of neutralizing antibody to the homologous strain of HIV tested by the protection of MT-2 cells from virus-induced killing as measured by neutral red uptake. Titers of neutralizing antibody raised by the gp120-expressing DNAs were at the background of the assay.

The results of this study showed that fusions of HIV-1 Env to three copies of murine C3d enhanced the antibody response to Env in vaccinated mice. Mice vaccinated with any of the three DNA plasmids expressing sgp120 sequence had low or undetectable levels of antibody after 4 vaccinations (28 weeks post-prime). In contrast, mice vaccinated with DNA expressing the fusion of sgp120 and 3C3d proteins elicited a faster onset of antibody (3 vaccinations), as well as higher levels of antibodies.

In contrast to the enhancement of antibody titers and avidity maturation of antibodies to Env, the amount of neutralizing-antibody elicited in the vaccinated mice was low. Mice vaccinated with plasmids expressing sgp120 had low levels of neutralizing antibody that were only modestly increased in mice vaccinated with sgp120-3C3d expressing plasmids. However, the levels of neutralizing antibodies did apparently increase after the fourth immunization. The poor titers of neutralizing antibody could have reflected an inherent poor ability of the sgp120-3C3d fusion protein to raise neutralizing antibody because of the failure to adequately expose neutralizing epitopes to responding B cells. The intrinsic high backgrounds for HIV-1 neutralization assays in mouse sera also may have contributed to the poor neutralization titers.

The results demonstrate the effectiveness of C3d-fusions as a molecular adjuvant in enhancing antibody production and enhancing antibody maturation. In addition, the neutralizing antibody response to Env was modestly increased in mice vaccinated with C3d-fusion vaccines. Similar to results seen in Examples 17 and 18, using secreted versions of HA from measles and influenza viruses, C3d-enhanced antibody responses were achieved with plasmids expressing only half as much protein as plasmids expressing non-fused sgp120.

## REFERENCES

- Amara, R., et al. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA Vaccine. Submitted.
- Andre, S., Seed, B., Eberle, J., Schraut, W., Bultmann, A., and Haas, J. (1998). Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol* 72(2), 1497503.
- Asakura, Y., Hinkula, J., Leandersson, A. C., Fukushima, J., Okuda, K., and Wahren, B. (1997). Induction of HIV-1 specific mucosal immune responses by DNA vaccination. *Scand J Immunol* 46(4), 326-30.

Bachmann M F, Zinkernagel R M. Neutralizing antiviral B cell responses. *Annu Rev Immunol* 1997; 15:235-70.

Barouch. D. H. et al., 2000. *Science* 290: 486-92.

Barry, M. A., Lai, W. C., and Johnston, S. A. (1995). Protection against mycoplasma infection using expression library immunization. *Nature* 377(6550), 632-5.

Berger E A (1997). HIV entry and tropism: the chemokine receptor connection. *AIDS* 11 Suppl A:S3-16

Bohm, W., Kuhrober, A., Paier, T., Mertens, T., Reimann, J., and Schirmbeck, R. (1996). DNA vector constructs that prime hepatitis B surface antigen-specific cytotoxic T lymphocyte and antibody responses in mice after intramuscular injection. *J Immunol Methods* 193(1), 29-40.

Bohm, W., Mertens, T., Schirmbeck, R., and Reimann, J. (1998). Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses. *Vaccine* 16(9-10), 949-54.

Bolivar F, Rodriguez R L, Greene P J, Betlach M C, Heyneker H L, Boyer H W (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2(2):95-113

Boyer, J. D., Ugen, K. E., Wang, B., Agadjanyan, M., Gilbert, L., Bagarazzi, M. L., Chattergoon, M., Frost, P., Javadian, A., Williams, W. V., Refaeli, Y., Ciccarelli, R. B., McCallus, D., Coney, L., and Weiner, D. B. (1997). Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. *Nat Med* 3(5), 526-32.

Boyle, J. S., Koniaras, C., and Lew, A. M. (1997). Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int Immunol* 9(12), 1897-906.

Burton, D. R., and Montefiori, D. C. (1997). The antibody response in HIV-1 infection [see comments]. *Aids* 11(Suppl A), S87-98.

Calarota, S., Bratt, G., Nordlund, S., Hinkula, J., Leandersson, A. C., Sandstrom, E., and Wahren, B. (1998). Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* 351(9112), 1320-5.

Cardoso, A. I., Blixenkrone-Moller, M., Fayolle, J., Liu, M., Buckland, R., and Wild, F. T. Immunization with plasmid DNA encoding for the measles virus hemagglutinin and nucleoprotein leads to humoral and cell-mediated immunity. 1998. *Virology*. 225:293-299.

Chapman B S, Thayer R M, Vincent K A, Haigwood N L. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Research* 1991; 19(14):3979-86.

Chen, S. C., Jones, D. H., Fynan, E. F., Farrar, G. H., Clegg, J. C., Greenberg, H. B., and Herrmann, J. E. (1998a). Protective immunity induced by oral immunization with a rotavirus; DNA vaccine encapsulated in microparticles. *J Virol* 72(7), 5757-61.

Chun. T. W. et al., 1998. *Proc Natl Acad Sci U S A* 95: 8869-73.

Collman R, Balliet J W, Gregory S A, Friedman H, Kolson D L, Nathanson N, et al. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J Virol* 1992; 66(12):7517-21.

Condon C, Watkins S C, Celluzzi C M, Thompson K, Falo L D, Jr. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* 1996; 2(10):1122-8.

Corr M, Lee D J, Carson D A, Tighe H. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J Exp Med* 1996; 184(4):1555-60.

Dempsey P W, Allison M E, Akkaraju S, Goodnow C C, Fearon D T (1996). C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271:348-50.

Egan, M. A. et al., (2000) *J Virol* 74: 7485-95.

Evans D T, O'Connor D H, Jing P, Dzuris J L, Sidney J, da Silva J, et al. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat Med* 1999; 5(11): 1270-6.

Feltquate, D. M., Heaney, S., Webster, R. G., and Robinson, H. L. (1997). Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *Journal of Immunology* 158(5), 2278-84.

Fomsgaard, A., Nielsen, H. V., Bryder, K., Nielsen, C., Machuca, K., Bruun, L., Hansen, J., and Buus, S. (1998). Improved humoral and cellular immune responses against the gp120 V3 loop of HIV-1 following genetic immunization with a chimeric DNA vaccine encoding the V3 inserted into the hepatitis B surface antigen. *Scand J Immunol* 47(4), 289-95.

Fu T M, Ulmer J B, Caulfield M J, Deck R R, Friedman A, Wang S, et al. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol Med* 1997; 3(6):362-71.

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. (1993). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci USA* 90(24), 11478-82.

Gromkowski, S. H., Nabel, G. J., and et al. (1995). Cancer gene therapy using plasmid DNA: pharmacokinetic study of DNA following injection in mice [see comments]. *Hum Gene Ther* 6(5), 553-64.

Hakim, I., Levy, S., and Levy, R. (1996). A nine-amino acid peptide from IL-1 beta augments antitumor immune responses induced by protein and DNA vaccines. *J Immunol* 157(12), 5503-11.

Hanke T, Samuel R V, Blanchard T J, Neumann V C, Allen T M, Boyson J E, Sharpe S A, Cook N, Smith G L, Watkins D I, Cranage M P, McMichael (1999). Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J Virol* 73(9):7524-32

Hanke, T., Blanchard, T. J., Schneider, J., Hannan, C. M., Becker, M., Gilbert S. C., Hill, A. V., Smith, G. L., and McMichael, A. (1998a). Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine* 16(5), 439-45.

Hanke, T., Schneider, J., Gilbert S. C., Hill, A. V., and McMichael, A. (1998b). DNA multi-CTL epitope vaccines for EIIV and Plasmodium falciparum: immunogenicity in mice. *Vaccine* 16(4), 426-35.

Hartikka, J., Sawdey, M., Comefert-Jensen, F., Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H. L., Meek, J., Marquet, M., Hobart, P., Norman, J., and Manthorpe, M. (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther* 7(10), 1205-17.

Inchauspe, G., Vitvitski, L., Major, M. E., Jung, G., Spengler, U., Maisonnas, M., and Trepo, C. (1997). Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C

virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. *DNA Cell Biol* 16(2), 185-95.

Iwasaki A, Torres C A, Ohashi P S, Robinson H L, Barber B H. (1997b) The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J Immunol* 159(1):11-4.

Iwasaki, A., Stiernholm, B. J., Chan, A. K., Berinstein, N. L., and Barber, B. H. (1997a). Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J Immunol* 158(10), 4591-601.

Jones, D. H., Corris, S., McDonald, S., Clegg, J. C., and Farrar, G. H. (1997). Poly(DL-lactide-co-glycolide) encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* 15(8), 814-7.

Kawabata, K., Takakura, Y., and Hashida, M. (1995). The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm Res* 12(6), 825-30.

Kent S. J., Zhao, A., Best, S. J., Chandler, J. D., Boyle, D. B., and Ramshaw, I. A. (1998). Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type I vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J Virol* 72(12), 10180-8.

Kuroda M J, Schmitz J E, Barouch D H, Craiu A, Allen T M, Sette A, et al. Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus-infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I-peptide complex. *Journal of Experimental Medicine* 1998; 187(9):1373-81.

Letvin, N. L., Montefiori, D. C., Yasutomi, Y., Perry, H. C., Davies, M. E., Lekutis, C., Alroy, M., Freed, D. C., Lord, C. I., Handt, L. K., Liu, M. A., and Shiver, J. W. (1997). Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proceedings of the National Academy of Sciences of the United States of America* 94(17), 9378-83.

Lew, D., Parker, S. E., Latimer, T., Abai, A. M., Kuwahara-Rundell, A., Doh, S. G., Yang, Z. Y., Laface, D., Gromkowski, S. H., and Nabel, G. J., et al. (1995). Cancer gene therapy using plasmid DNA: Pharmacokinetic study of DNA following injection in mice [see comments]. *Hum. Gene Ther.* 6:553.

Li J, Lord C I, Haseltine W, Letvin N L, Sodroski J. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. *Journal of Acquired Immune Deficiency Syndromes* 1992; 5(7):639-46.

Livingston, J. B., Lu, S., Robinson, H. L., and Anderson, D. J. (1995). The induction of mucosal immunity in the female genital tract using gene-gun technology. Part 1: Antigen expression. *Annals of the New York Academy of Sciences* 772, 265-7.

Lu, S., Manson, K., Wyand, M., and Robinson, H. L. (1997). SIV DNA vaccine trial in macaques: post-challenge necropsy in vaccine and control groups. *Vaccine* 15(g), 920-3.

MacGregor, R. R., Gluckman, S., Lacy, K., Kaniefski, B., Boyer, J., Wang, B., Bagarazzi, M., William, W. V., Francher, D., Ginsberg, R., Higgins, T., and Weiner, D. (1996). First human trial of a facilitated DNA plasmid vaccine for HIV-1: safety and host response. *Int Conf AIDS* 11(2), 23 (abstract no. We.B.293).

Maecker, H. T., Umetsu, D. T., De Kruffyff, R. H., and Levy, S. (1997). DNA vaccination with cytokine fusion constructs biases the immune response to ovalbumin. *Vaccine* 15(15), 1687-96.

Maecker, H. T., Umetsu, D. T., De Kruffyff, R. H., and Levy, S. (1998). Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via class II MHC. *J Immunol* 161(12), 6532-6.

Manthorpe, M., Comefert-Jensen, F., Hartikka, J., Feigner, J., Rundell, A., Margalith, M., and Dwarki, V. (1993). Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum Gene Ther* 4(4), 419-31.

McCluskie, M. J., Chu, Y., Xia, J. L., Jessee, J., Gebyehu, G., and Davis, H. L. (1998). Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA [In Process Citation]. *Antisense Nucleic Acid Drug Dev* 8(5), 401-14.

Montefiori, D. C., W. E. Robinson, S. S. Schuffman, and W. M. Mitchell, 1988. Evaluation of antiviral drugs and neutralizing antibodies to HIV by a rapid and sensitive microtiter infection assay. *J. Clin. Microbiol.* 26:231-237.

Montgomery, D. L., Shiver, J. W., Leander, K. R., Perry, H. C., Friedman, A., Martinez, D., Ulmer, J. B., Donnelly, J. J., and Liu, M. A. (1993). Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol* 12(9), 777-83.

Moore, J. P., and Ho, D. D. (1995). HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells. *Aids* 9(Suppl A), S 117-36.

Murali-Krishna K, Altman J D, Suresh M, Sourdive D J, Zajac A J, Miller J D, et al. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 1998; 8(2):177-87.

Ourmanov, I. et al. 2000. *J Virol* 74: 2740-51.

Pertmer, T. M., Eisenbraun, M. D., McCabe, D., Prayaga, S. K., Fuller, D. H., and Haynes, J. R. (1995). Gene gunbased nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 13(15), 1427-30.

Pertmer, T. M., Roberts, T. R., and Haynes, J. R. (1996). Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J Virol* 70(9), 6119-25.

Pertmer, T. M. and Robinson, H. L. (In press). Studies on antibody responses following neonatal immunization with influenza hemagglutinin DNA or protein. *Virology*.

Poignard P, Sabbe R, Picchio G R, Wang M, Gulizia R J, Katinger H, Parren P W, Mosier D E, Burton D R (1999). Neutralizing antibodies have limited effects on the control of established HIV-1 infection in vivo. *Immunity* 10(4): 431-8

Porgador A, Irvine K R, Iwasaki A, Barber B H, Restifo N P, Germain R N. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med* 1998; 188(6):1075-82.

Reimann K A, Li J T, Veazey R, Halloran M, Park I W, Karlsson G B, et al. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *Journal of Virology* 1996; 70(10):6922-8.



Reimann K A, Li J T, Voss G, Lekutis C, Tenner-Racz K, Racz P, et al. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. *Journal of Virology* 1996; 70(5):3198-206.

Richmond, J. F., Lu, S., Santoro, J. C., Weng, J., Hu, S. L., Montefiori, D. C., and Robinson, H. L. (1998). Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. *J Virol* 72(11), 9092-9100.

Robinson, H. L. and Pertmer, T. M. (2000). DNA vaccines: Basic studies and applications. In *Advances in Virus Research*. Academic Press., Vol. 53 (in press).

Robinson, H. L., Montefiori, D. C., Johnson, R. P., Manson, K. H., Kalish, M. L., Lifson, J. D., Rizvi, T. A., Lu, S., Hu, S. L., Mazzara, G. P., Panicali, D. L., Herndon, J. G., Glickman, R., Candido, M. A., Lydy, S. L., Wand, M. S., and McClure, H. M. (1999). Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat. Med.* 5:526.

Robinson, H. L., and Pertmer, T. M. (1998). Nucleic Acid Immunizations. In "Current Protocols in Immunology" (R. Coico, Ed.), Vol. 1, pp. 2.14.1-2.14.19. 3 vols. John Wiley & Sons, Inc., New York.

Robinson, H. L., Ginsberg, H. S., Davis, H. L., Johnston, S. A., and Liu, M. A. (1997). The Scientific Future of DNA for Immunization. *American Academy of Microbiology*. May 31-Jun. 2-1996.

Robinson, H. L., Hunt, L. A., and Webster, R. G. (1993). Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine* 11(9), 957-60.

Rodriguez, F., Zhang, J., and Whitton, J. L. (1997). DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J Virol* 71(11), 8497-503.

Ross, T. M., Y. Xu, R. A. Bright, and H. L. Robinson. C3d enhancement of antibodies to Hemagglutinin accelerates protection against influenza virus challenge. *Nat. Immunol.* 2000. 1:127-131.

Ross, T. M., Y. Xu, T. D. Green, D. C. Montefiori, and H. L. Robinson. 2001. Enhanced Avidity maturation of Antibody to Human Immunodeficiency Virus envelope: DNA Vaccination with gp120-C3d Fusion Proteins. *AIDS Res. Human Retro*. In press.

Sasaki, S., Hamajima, K., Fukushima, J., Ihata, A., Ishii, N., Gorai, I., Hirahara, F., Mohri, H., and Okuda, K. (1998b). Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type I with a DNA-monophosphoryl lipid A adjuvant vaccine. *Infect Immun* 66(2), 823-6.

Schneider, J., Gilbert, S. C., Blanchard, T. J., Hanke, T., Robson, K. J., Hannan, C. M., Becker, M., Sinden, R. Smith, G. L., and Hill, A. V. (1998). Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat Med* 4(4), 397-402.

Scholtissek S, Grosse F. A cloning cartridge of lambda t(o) terminator. *Nucleic Acids Res* 1987; 15(7):3185.

Sizemore, D. R., Branstrom, A. A., and Sadoff, J. C. (1997). Attenuated bacteria as a DNA delivery vehicle for DNA-

mediated immunization. *Science* 270(5234), 299-302.

Staprans, S., B. Corliss, J. Guthrie, M. B. Feinberg, in *Viral Genome Methods* K. Adolph, Ed. (CRC Press, Boca Raton, Fla., 1996) pp. 167-184.

Subbarao S, Schochetman G. Genetic variability of HIV-1. *Aids* 1996; 10(Suppl A):S13-23.

Sutcliffe, et al. 1978. *Cold Spring Harbor Quant. Biol.* 43:77-90

Tang, D. C., De Vit, M., and Johnston, S. A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* 356(6365), 152-4.

Thomson, S. A., Sherritt, M. A., Medveczky, J., Elliott, S. L., Moss, D. J., Fernando, G. J., Brown, L. E., and Suhrbier, A. (1998). Delivery of multiple CD8 cytotoxic T cell epitopes by DNA vaccination. *J Immunol* 160(4), 1717-23.

Tobery, T. W., and Siliciano, R. F. (1997). Targeting of FHV-I antigens for rapid intracellular degradation enhances cytotoxic T lymphocyte (CTL) recognition and the induction of de novo CTL responses in vivo after immunization. *J Exp Med* 185(5), 909-20.

Torres C A, Yang K, Mustafa F, Robinson H L (2000). DNA immunization: effect of secretion of DNA-expressed hemagglutinins on antibody responses. *Vaccine* 18: 805-14

Torres C A, Iwasaki A, Barber B H, Robinson H L. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J Immunol* 1997; 158(10): 4529-32.

Uchijima, M., Yoshida, A., Nagata, T., and Koide, Y. (1998). Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *J Immunol* 161(10), 5594-9.

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., De Witt, C. M., Friedman, A., and et al. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259(5102), 1745-9.

Wild, J., Gruner, B., Metzger, K., Kuhrober, A., Pudollek, H. P., Hauser, H., Schirmbeck, R., and Reimann, J. (1998). Polyvalent vaccination against hepatitis B surface and core antigen using a dicistronic expression plasmid. *Vaccine* 16(4), 353-60.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* 247(4949 Pt 1), 1465-8.

Wu, Y., and Kipps, T. J. (1997). Deoxyribonucleic acid vaccines encoding antigens with rapid proteasomedependent degradation are highly efficient inducers of cytolytic T lymphocytes. *J Immunol* 159(12), 603743.

Xiang, Z., and Ertl, H. C. (1995). Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunology* 2(2), 129-35.

Yamamoto S, Folks T M, Heneine W. Highly sensitive qualitative and quantitative detection of reverse transcriptase activity: optimization, validation, and comparative analysis with other detection systems. *J Virol Methods* 1996; 61(1-2): 135-43.



## SEQUENCE LISTING

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<212> TYPE: DNA
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<221> NAME/KEY: promoter
<222> LOCATION: (1)...(682)

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<223> OTHER INFORMATION: cytomegalovirus intermediate early promoter

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<210> SEQ ID NO 3
<211> LENGTH: 3893
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: vaccine vector pGA3
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)...(690)
<223> OTHER INFORMATION: cytomegalovirus intermediate early promoter
<400> SEQUENCE: 3

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<210> SEQ ID NO 4
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: construct of vaccine vector pGA2 and insert JS2
expressing clade HIV-1 VL

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<400> SEQUENCE: 4

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<210> SEQ ID NO 6
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic oligonucleotide

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<400> SEQUENCE: 6

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<210> SEQ ID NO 7
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 7

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<210> SEQ ID NO 8

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<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 8

cgtggatggc gtatcgatgc gatctgacgg 30

<210> SEQ ID NO 9  
<211> LENGTH: 29  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 9

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<210> SEQ ID NO 10  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 10

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<210> SEQ ID NO 11  
<211> LENGTH: 17  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 11

gggcaggagt gctagcc 17

<210> SEQ ID NO 12  
<211> LENGTH: 29  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 12

ccacactact ttcggaccgc tagccacc 29

<210> SEQ ID NO 13  
<211> LENGTH: 32  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 13

ggttaagagc ttcaatagcg gcaaagaagg gc 32

<210> SEQ ID NO 14  
<211> LENGTH: 32  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 14

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gcccttcttt gccgtattg aagctcttaa cc

32

<210> SEQ ID NO 15  
<211> LENGTH: 27  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 15

gggcagctgg aaaagcggaa aggaagg

27

<210> SEQ ID NO 16  
<211> LENGTH: 27  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 16

ccttcctttc cgcttttcca gctgcc

27

<210> SEQ ID NO 17  
<211> LENGTH: 44  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 17

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44

<210> SEQ ID NO 18  
<211> LENGTH: 44  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 18

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44

<210> SEQ ID NO 19  
<211> LENGTH: 33  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 19

ggggaaattg aataccgcaa gtcagattta ccc

33

<210> SEQ ID NO 20  
<211> LENGTH: 33  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 20

gggtaaatct gacttgcggg attcaatttc ccc

33

<210> SEQ ID NO 21  
<211> LENGTH: 40  
<212> TYPE: DNA



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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 21
ccctaactaa cacaacaaat cagaaaaactc agttacaagc
40

<210> SEQ ID NO 22
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 22
gcttgtaact gagttttctg atttggtgtg ttagttaggg
40

<210> SEQ ID NO 23
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 23
ggcaactaaa ggaagctcta ttagccacag gagg
34

<210> SEQ ID NO 24
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 24
gctcctgtgg ctaatagagc ttccttagt tgcc
34

<210> SEQ ID NO 25
<211> LENGTH: 512
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protein encoded by construct of vaccine vector
        pGA2 and insert JS2 expressing clade HIV-1 VL

<400> SEQUENCE: 25
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 1             5             10            15
Glu Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys Lys Tyr Lys Leu Lys
 20            25            30
His Ile Val Trp Ala Ser Arg Glu Leu Glu Arg Phe Ala Val Asn Pro
 35            40            45
Gly Leu Leu Glu Thr Ser Glu Gly Cys Arg Gln Ile Leu Gly Gln Leu
 50            55            60
Gln Pro Ser Leu Gln Thr Gly Ser Glu Glu Leu Arg Ser Leu Tyr Asn
 65            70            75            80
Thr Val Ala Thr Leu Tyr Cys Val His Gln Arg Ile Glu Ile Lys Asp
 85            90            95
Thr Lys Glu Ala Leu Asp Lys Ile Glu Glu Glu Gln Asn Lys Ser Lys
100           105           110
Lys Lys Ala Gln Gln Ala Ala Asp Thr Gly His Ser Ser Gln Val
115           120           125

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Ser	Gln	Asn	Tyr	Pro	Ile	Val	Gln	Asn	Ile	Gln	Gly	Gln	Met	Val	His
130						135					140				
Gln	Ala	Ile	Ser	Pro	Arg	Thr	Leu	Asn	Ala	Trp	Val	Lys	Val	Val	Glu
145					150					155					160
Glu	Lys	Ala	Phe	Ser	Pro	Glu	Val	Ile	Pro	Met	Phe	Ser	Ala	Leu	Ser
				165					170					175	
Glu	Gly	Ala	Thr	Pro	Gln	Asp	Leu	Asn	Thr	Met	Leu	Asn	Thr	Val	Gly
			180					185						190	
Gly	His	Gln	Ala	Ala	Met	Gln	Met	Leu	Lys	Glu	Thr	Ile	Asn	Glu	Glu
		195					200						205		
Ala	Ala	Glu	Trp	Asp	Arg	Val	His	Pro	Val	His	Ala	Gly	Pro	Ile	Ala
		210					215					220			
Pro	Gly	Gln	Met	Arg	Glu	Pro	Arg	Gly	Ser	Asp	Ile	Ala	Gly	Thr	Thr
225					230					235					240
Ser	Thr	Leu	Gln	Glu	Gln	Ile	Gly	Trp	Met	Thr	Asn	Asn	Pro	Pro	Ile
				245					250					255	
Pro	Val	Gly	Glu	Ile	Tyr	Lys	Arg	Trp	Ile	Ile	Leu	Gly	Leu	Asn	Lys
			260					265						270	
Ile	Val	Arg	Met	Tyr	Ser	Pro	Thr	Ser	Ile	Leu	Asp	Ile	Arg	Gln	Gly
		275					280						285		
Pro	Lys	Glu	Pro	Phe	Arg	Asp	Tyr	Val	Asp	Arg	Phe	Tyr	Lys	Thr	Leu
		290				295						300			
Arg	Ala	Glu	Gln	Ala	Ser	Gln	Glu	Val	Lys	Asn	Trp	Met	Thr	Glu	Thr
305					310					315					320
Leu	Leu	Val	Gln	Asn	Ala	Asn	Pro	Asp	Cys	Lys	Thr	Ile	Leu	Lys	Ala
				325					330					335	
Leu	Gly	Pro	Ala	Ala	Thr	Leu	Glu	Glu	Met	Met	Thr	Ala	Cys	Gln	Gly
			340					345					350		
Val	Gly	Gly	Pro	Gly	His	Lys	Ala	Arg	Val	Leu	Ala	Glu	Ala	Met	Ser
		355					360						365		
Gln	Val	Thr	Asn	Thr	Ala	Thr	Ile	Met	Met	Gln	Arg	Gly	Asn	Phe	Arg
		370				375						380			
Asn	Gln	Arg	Lys	Met	Val	Lys	Ser	Phe	Asn	Ser	Gly	Lys	Glu	Gly	His
385					390					395					400
Thr	Ala	Arg	Asn	Cys	Arg	Ala	Pro	Arg	Lys	Lys	Gly	Ser	Trp	Lys	Ser
				405					410					415	
Gly	Lys	Glu	Gly	His	Gln	Met	Lys	Asp	Cys	Thr	Glu	Arg	Gln	Ala	Asn
			420					425					430		
Phe	Leu	Gly	Lys	Ile	Trp	Pro	Ser	Tyr	Lys	Gly	Arg	Pro	Gly	Asn	Phe
		435				440							445		
Leu	Gln	Ser	Arg	Pro	Glu	Pro	Thr	Ala	Pro	Pro	Phe	Leu	Gln	Ser	Arg
		450				455						460			
Pro	Glu	Pro	Thr	Ala	Pro	Pro	Glu	Glu	Ser	Phe	Arg	Ser	Gly	Val	Glu
465					470					475					480
Thr	Thr	Thr	Pro	Pro	Gln	Lys	Gln	Glu	Pro	Ile	Asp	Lys	Glu	Leu	Tyr
				485					490					495	
Pro	Leu	Thr	Ser	Leu	Arg	Ser	Leu	Phe	Gly	Asn	Asp	Pro	Ser	Ser	Gln
			500					505					510		

&lt;210&gt; SEQ ID NO 26

&lt;211&gt; LENGTH: 739

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: protein encoded by construct of vaccine vector

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pGA2 and insert JS2 expressing clade HIV-1 VL

&lt;400&gt; SEQUENCE: 26

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Phe Phe Arg Glu Asp Leu Ala Phe Leu Gln Gly Lys Ala Arg Glu Phe
 1         5         10        15
Ser Ser Glu Gln Thr Arg Ala Asn Ser Pro Thr Ile Ser Ser Glu Gln
 20        25        30
Thr Gly Ala Asn Ser Pro Thr Arg Arg Glu Leu Gln Val Trp Gly Arg
 35        40        45
Asp Asn Asn Ser Pro Ser Glu Ala Gly Ala Asp Arg Gln Gly Thr Val
 50        55        60
Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp Gln Arg Pro Leu Val Thr
 65        70        75        80
Ile Lys Ile Gly Gly Gln Leu Lys Glu Ala Leu Leu Asp Thr Gly Ala
 85        90        95
Asp Asp Thr Val Leu Glu Glu Met Ser Leu Pro Gly Arg Trp Lys Pro
100       105       110
Lys Met Ile Gly Gly Ile Gly Gly Phe Ile Lys Val Arg Gln Tyr Asp
115       120       125
Gln Ile Leu Ile Glu Ile Cys Gly His Lys Ala Ile Gly Thr Val Leu
130       135       140
Val Gly Pro Thr Pro Val Asn Ile Ile Gly Arg Asn Leu Leu Thr Gln
145       150       155       160
Ile Gly Cys Thr Leu Asn Phe Pro Ile Ser Pro Ile Glu Thr Val Pro
165       170       175
Val Lys Leu Lys Pro Gly Met Asp Gly Pro Lys Val Lys Gln Trp Pro
180       185       190
Leu Thr Glu Glu Lys Ile Lys Ala Leu Val Glu Ile Cys Thr Glu Met
195       200       205
Glu Lys Glu Gly Lys Ile Ser Lys Ile Gly Pro Glu Asn Pro Tyr Asn
210       215       220
Thr Pro Val Phe Ala Ile Lys Lys Lys Asp Ser Thr Lys Trp Arg Lys
225       230       235       240
Leu Val Asp Phe Arg Glu Leu Asn Lys Arg Thr Gln Asp Phe Trp Glu
245       250       255
Val Gln Leu Gly Ile Pro His Pro Ala Gly Leu Lys Lys Lys Lys Ser
260       265       270
Val Thr Val Leu Asp Val Gly Asp Ala Tyr Phe Ser Val Pro Leu Asp
275       280       285
Glu Asp Phe Arg Lys Tyr Thr Ala Phe Thr Ile Pro Ser Ile Asn Asn
290       295       300
Glu Thr Pro Gly Ile Arg Tyr Gln Tyr Asn Val Leu Pro Gln Gly Trp
305       310       315       320
Lys Gly Ser Pro Ala Ile Phe Gln Ser Ser Met Thr Lys Ile Leu Glu
325       330       335
Pro Phe Lys Lys Gln Asn Pro Asp Ile Val Ile Tyr Gln Tyr Met Asn
340       345       350
Asp Leu Tyr Val Gly Ser Asp Leu Glu Ile Gly Gln His Arg Thr Lys
355       360       365
Ile Glu Glu Leu Arg Gln His Leu Leu Arg Trp Gly Leu Thr Thr Pro
370       375       380
Asp Lys Lys His Gln Lys Glu Pro Pro Phe Leu Trp Met Gly Tyr Glu
385       390       395       400

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Leu His Pro Asp Lys Trp Thr Val Gln Pro Ile Val Leu Pro Glu Lys  
 405 410 415  
 Asp Ser Trp Thr Val Asn Asp Ile Gln Lys Leu Val Gly Lys Leu Asn  
 420 425 430  
 Thr Ala Ser Gln Ile Tyr Pro Gly Ile Lys Val Arg Gln Leu Cys Lys  
 435 440 445  
 Leu Leu Arg Gly Thr Lys Ala Leu Thr Glu Val Ile Pro Leu Thr Glu  
 450 455 460  
 Glu Ala Glu Leu Glu Leu Ala Glu Asn Arg Glu Ile Leu Lys Glu Pro  
 465 470 475 480  
 Val His Gly Val Tyr Tyr Asp Pro Ser Lys Asp Leu Ile Ala Glu Ile  
 485 490 495  
 Gln Lys Gln Gly Gln Gly Gln Trp Thr Tyr Gln Ile Tyr Gln Glu Pro  
 500 505 510  
 Phe Lys Asn Leu Lys Thr Gly Lys Tyr Ala Arg Met Arg Gly Ala His  
 515 520 525  
 Thr Asn Asp Val Lys Leu Leu Thr Glu Ala Val Gln Lys Ile Thr Thr  
 530 535 540  
 Glu Ser Ile Val Ile Trp Gly Lys Thr Pro Lys Phe Lys Leu Pro Ile  
 545 550 555 560  
 Gln Lys Glu Thr Trp Glu Thr Trp Trp Thr Glu Tyr Trp Gln Ala Thr  
 565 570 575  
 Trp Ile Pro Glu Trp Glu Phe Val Asn Thr Pro Pro Leu Val Lys Leu  
 580 585 590  
 Trp Tyr Gln Leu Glu Lys Glu Pro Ile Val Gly Ala Glu Thr Phe Tyr  
 595 600 605  
 Val Asp Gly Ala Ala Asn Arg Glu Thr Lys Leu Gly Lys Ala Gly Tyr  
 610 615 620  
 Val Thr Asn Lys Gly Arg Gln Lys Val Val Pro Leu Thr Asn Thr Thr  
 625 630 635 640  
 Asn Gln Lys Thr Gln Leu Gln Ala Ile Tyr Leu Ala Leu Gln Asp Ser  
 645 650 655  
 Gly Leu Glu Val Asn Ile Val Thr Asp Ser Gln Tyr Ala Leu Gly Ile  
 660 665 670  
 Ile Gln Ala Gln Pro Asp Lys Ser Glu Ser Glu Leu Val Asn Gln Ile  
 675 680 685  
 Ile Glu Gln Leu Ile Lys Lys Glu Lys Val Tyr Leu Ala Trp Val Pro  
 690 695 700  
 Ala His Lys Gly Ile Gly Gly Asn Glu Gln Val Asp Lys Leu Val Ser  
 705 710 715 720  
 Ala Gly Ile Arg Lys Ile Leu Phe Leu Asp Gly Ile Asp Lys Ala Gln  
 725 730 735  
 Asp Glu His

&lt;210&gt; SEQ ID NO 27

&lt;211&gt; LENGTH: 72

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

 <223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
 pGA2 and insert JS2 expressing clade HIV-1 VL

&lt;400&gt; SEQUENCE: 27

Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser  
 1 5 10 15

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Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe  
                   20                  25                  30  
 His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly  
           35                  40                  45  
 Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Asp Ser Gln Thr  
       50                  55                  60  
 His Gln Val Ser Leu Ser Lys Gln  
   65                  70

<210> SEQ ID NO 28  
 <211> LENGTH: 25  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
                           pGA2 and insert JS2 expressing clade HIV-1 VL

<400> SEQUENCE: 28

Met Ala Gly Arg Ser Gly Asp Ser Asp Glu Asp Leu Leu Lys Thr Val  
   1                  5                  10                  15  
 Arg Leu Ile Lys Phe Leu Tyr Gln Ser  
           20                  25

<210> SEQ ID NO 29  
 <211> LENGTH: 852  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
                           pGA2 and insert JS2 expressing clade HIV-1 VL

<400> SEQUENCE: 29

Met Lys Val Lys Gly Ile Arg Lys Asn Tyr Gln His Leu Trp Lys Trp  
   1                  5                  10                  15  
 Gly Ile Met Leu Leu Gly Met Leu Met Ile Cys Ser Ala Val Glu Asn  
           20                  25                  30  
 Leu Trp Val Thr Val Tyr Tyr Gly Val Pro Val Trp Lys Glu Ala Thr  
       35                  40                  45  
 Thr Thr Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr Asp Thr Glu Val  
       50                  55                  60  
 His Asn Val Trp Ala Thr His Ala Cys Val Pro Thr Asp Pro Asn Pro  
   65                  70                  75                  80  
 Gln Glu Val Val Leu Glu Asn Val Thr Glu Asn Phe Asn Met Trp Lys  
           85                  90                  95  
 Asn Asn Met Val Glu Gln Met His Glu Asp Ile Ile Ser Leu Trp Asp  
       100                  105                  110  
 Gln Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val Thr Leu  
       115                  120                  125  
 Asn Cys Thr Asp Leu Arg Asn Val Thr Asn Ile Asn Asn Ser Ser Glu  
       130                  135                  140  
 Gly Met Arg Gly Glu Ile Lys Asn Cys Ser Phe Asn Ile Thr Thr Ser  
   145                  150                  155                  160  
 Ile Arg Asp Lys Val Lys Lys Asp Tyr Ala Leu Phe Tyr Arg Leu Asp  
           165                  170                  175  
 Val Val Pro Ile Asp Asn Asp Asn Thr Ser Tyr Arg Leu Ile Asn Cys  
       180                  185                  190  
 Asn Thr Ser Thr Ile Thr Gln Ala Cys Pro Lys Val Ser Phe Glu Pro  
       195                  200                  205

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Ile	Pro	Ile	His	Tyr	Cys	Thr	Pro	Ala	Gly	Phe	Ala	Ile	Leu	Lys	Cys
210						215					220				
Lys	Asp	Lys	Lys	Phe	Asn	Gly	Thr	Gly	Pro	Cys	Lys	Asn	Val	Ser	Thr
225					230					235					240
Val	Gln	Cys	Thr	His	Gly	Ile	Arg	Pro	Val	Val	Ser	Thr	Gln	Leu	Leu
				245					250					255	
Leu	Asn	Gly	Ser	Leu	Ala	Glu	Glu	Glu	Val	Val	Ile	Arg	Ser	Ser	Asn
		260						265					270		
Phe	Thr	Asp	Asn	Ala	Lys	Asn	Ile	Ile	Val	Gln	Leu	Lys	Glu	Ser	Val
		275					280					285			
Glu	Ile	Asn	Cys	Thr	Arg	Pro	Asn	Asn	Asn	Thr	Arg	Lys	Ser	Ile	His
	290					295					300				
Ile	Gly	Pro	Gly	Arg	Ala	Phe	Tyr	Thr	Thr	Gly	Glu	Ile	Ile	Gly	Asp
305					310					315					320
Ile	Arg	Gln	Ala	His	Cys	Asn	Ile	Ser	Arg	Thr	Lys	Trp	Asn	Asn	Thr
				325					330					335	
Leu	Asn	Gln	Ile	Ala	Thr	Lys	Leu	Lys	Glu	Gln	Phe	Gly	Asn	Asn	Lys
			340					345					350		
Thr	Ile	Val	Phe	Asn	Gln	Ser	Ser	Gly	Gly	Asp	Pro	Glu	Ile	Val	Met
		355					360					365			
His	Ser	Phe	Asn	Cys	Gly	Gly	Glu	Phe	Phe	Tyr	Cys	Asn	Ser	Thr	Gln
	370					375					380				
Leu	Phe	Asn	Ser	Thr	Trp	Asn	Phe	Asn	Gly	Thr	Trp	Asn	Leu	Thr	Gln
385					390					395					400
Ser	Asn	Gly	Thr	Glu	Gly	Asn	Asp	Thr	Ile	Thr	Leu	Pro	Cys	Arg	Ile
			405					410						415	
Lys	Gln	Ile	Ile	Asn	Met	Trp	Gln	Glu	Val	Gly	Lys	Ala	Met	Tyr	Ala
		420						425					430		
Pro	Pro	Ile	Arg	Gly	Gln	Ile	Arg	Cys	Ser	Ser	Asn	Ile	Thr	Gly	Leu
		435					440					445			
Ile	Leu	Thr	Arg	Asp	Gly	Gly	Thr	Asn	Ser	Ser	Gly	Ser	Glu	Ile	Phe
	450					455					460				
Arg	Pro	Gly	Gly	Gly	Asp	Met	Arg	Asp	Asn	Trp	Arg	Ser	Glu	Leu	Tyr
465					470					475					480
Lys	Tyr	Lys	Val	Val	Lys	Ile	Glu	Pro	Leu	Gly	Val	Ala	Pro	Thr	Lys
			485					490						495	
Ala	Lys	Arg	Arg	Val	Val	Gln	Arg	Glu	Lys	Arg	Ala	Val	Gly	Thr	Ile
			500					505					510		
Gly	Ala	Met	Phe	Leu	Gly	Phe	Leu	Gly	Ala	Ala	Gly	Ser	Thr	Met	Gly
		515					520					525			
Ala	Ala	Ser	Ile	Thr	Leu	Thr	Val	Gln	Ala	Arg	Leu	Leu	Leu	Ser	Gly
	530					535					540				
Ile	Val	Gln	Gln	Gln	Asn	Asn	Leu	Leu	Arg	Ala	Ile	Glu	Ala	Gln	Gln
545					550					555					560
His	Leu	Leu	Gln	Leu	Thr	Val	Trp	Gly	Ile	Lys	Gln	Leu	Gln	Ala	Arg
			565					570						575	
Val	Leu	Ala	Leu	Glu	Arg	Tyr	Leu	Arg	Asp	Gln	Gln	Leu	Leu	Gly	Ile
			580					585					590		
Trp	Gly	Cys	Ser	Gly	Lys	Leu	Ile	Cys	Thr	Thr	Ala	Val	Pro	Trp	Asn
		595					600					605			
Ala	Ser	Trp	Ser	Asn	Lys	Thr	Leu	Asp	Met	Ile	Trp	Asp	Asn	Met	Thr
	610					615					620				
Trp	Met	Glu	Trp	Glu	Arg	Glu	Ile	Glu	Asn	Tyr	Thr	Gly	Leu	Ile	Tyr

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625	630	635	640
Thr Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Asp	645	650	655
Leu Leu Ala Leu Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe Asp Ile	660	665	670
Ser Asn Trp Leu Trp Cys Ile Lys Ile Phe Ile Met Ile Val Gly Gly	675	680	685
Leu Ile Gly Leu Arg Ile Val Phe Thr Val Leu Ser Ile Val Asn Arg	690	695	700
Val Arg Gln Gly Tyr Ser Pro Leu Ser Phe Gln Thr His Leu Pro Ala	705	710	715
Pro Arg Gly Pro Asp Arg Pro Glu Gly Ile Glu Glu Glu Gly Gly Asp	725	730	735
Arg Asp Arg Asp Arg Ser Val Arg Leu Val Asp Gly Ser Leu Ala Leu	740	745	750
Ile Trp Asp Asp Leu Arg Ser Leu Cys Leu Phe Ser Tyr His Arg Leu	755	760	765
Arg Asp Leu Leu Leu Ile Val Thr Arg Ile Val Glu Leu Leu Gly Arg	770	775	780
Arg Gly Trp Glu Ala Leu Lys Tyr Trp Trp Asn Leu Leu Gln Tyr Trp	785	790	795
Ser Gln Glu Leu Lys Asn Ser Ala Val Ser Leu Leu Asn Ala Thr Ala	805	810	815
Ile Ala Val Ala Glu Gly Thr Asp Arg Val Ile Glu Val Val Gln Gly	820	825	830
Ala Tyr Arg Ala Ile Arg His Ile Pro Arg Arg Ile Arg Gln Gly Leu	835	840	845
Glu Ile Leu Leu	850		

&lt;210&gt; SEQ ID NO 30

&lt;211&gt; LENGTH: 512

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
pGAI and vaccine insert expressing clade B HIV-1  
Gag-Pol

&lt;400&gt; SEQUENCE: 30

Met Gly Ala Arg Ala Ser Val Leu Ser Gly Gly Glu Leu Asp Arg Trp	1	5	10	15
Glu Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys Lys Tyr Lys Leu Lys	20	25	30	
His Ile Val Trp Ala Ser Arg Glu Leu Glu Arg Phe Ala Val Asn Pro	35	40	45	
Gly Leu Leu Glu Thr Ser Glu Gly Cys Arg Gln Ile Leu Gly Gln Leu	50	55	60	
Gln Pro Ser Leu Gln Thr Gly Ser Glu Glu Leu Arg Ser Leu Tyr Asn	65	70	75	80
Thr Val Ala Thr Leu Tyr Cys Val His Gln Arg Ile Glu Ile Lys Asp	85	90	95	
Thr Lys Glu Ala Leu Asp Lys Ile Glu Glu Glu Gln Asn Lys Ser Lys	100	105	110	
Lys Lys Ala Gln Gln Ala Ala Ala Asp Thr Gly His Ser Ser Gln Val	115	120	125	

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Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile Gln Gly Gln Met Val His
 130                135                140

Gln Ala Ile Ser Pro Arg Thr Leu Asn Ala Trp Val Lys Val Val Glu
145                150                155                160

Glu Lys Ala Phe Ser Pro Glu Val Ile Pro Met Phe Ser Ala Leu Ser
                165                170                175

Glu Gly Ala Thr Pro Gln Asp Leu Asn Thr Met Leu Asn Thr Val Gly
                180                185                190

Gly His Gln Ala Ala Met Gln Met Leu Lys Glu Thr Ile Asn Glu Glu
 195                200                205

Ala Ala Glu Trp Asp Arg Val His Pro Val His Ala Gly Pro Ile Ala
 210                215                220

Pro Gly Gln Met Arg Glu Pro Arg Gly Ser Asp Ile Ala Gly Thr Thr
225                230                235                240

Ser Thr Leu Gln Glu Gln Ile Gly Trp Met Thr Asn Asn Pro Pro Ile
                245                250                255

Pro Val Gly Glu Ile Tyr Lys Arg Trp Ile Ile Leu Gly Leu Asn Lys
                260                265                270

Ile Val Arg Met Tyr Ser Pro Thr Ser Ile Leu Asp Ile Arg Gln Gly
 275                280                285

Pro Lys Glu Pro Phe Arg Asp Tyr Val Asp Arg Phe Tyr Lys Thr Leu
 290                295                300

Arg Ala Glu Gln Ala Ser Gln Glu Val Lys Asn Trp Met Thr Glu Thr
305                310                315                320

Leu Leu Val Gln Asn Ala Asn Pro Asp Cys Lys Thr Ile Leu Lys Ala
                325                330                335

Leu Gly Pro Ala Ala Thr Leu Glu Glu Met Met Thr Ala Cys Gln Gly
                340                345                350

Val Gly Gly Pro Gly His Lys Ala Arg Val Leu Ala Glu Ala Met Ser
 355                360                365

Gln Val Thr Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Arg
 370                375                380

Asn Gln Arg Lys Met Val Lys Ser Phe Asn Ser Gly Lys Glu Gly His
385                390                395                400

Thr Ala Arg Asn Cys Arg Ala Pro Arg Lys Lys Gly Ser Trp Lys Ser
                405                410                415

Gly Lys Glu Gly His Gln Met Lys Asp Cys Thr Glu Arg Gln Ala Asn
 420                425                430

Phe Leu Gly Lys Ile Trp Pro Ser Tyr Lys Gly Arg Pro Gly Asn Phe
 435                440                445

Leu Gln Ser Arg Pro Glu Pro Thr Ala Pro Pro Phe Leu Gln Ser Arg
 450                455                460

Pro Glu Pro Thr Ala Pro Pro Glu Glu Ser Phe Arg Ser Gly Val Glu
465                470                475                480

Thr Thr Thr Pro Pro Gln Lys Gln Glu Pro Ile Asp Lys Glu Leu Tyr
                485                490                495

Pro Leu Thr Ser Leu Arg Ser Leu Phe Gly Asn Asp Pro Ser Ser Gln
 500                505                510

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&lt;210&gt; SEQ ID NO 31

&lt;211&gt; LENGTH: 739

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:



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<223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
pGAl and vaccine insert expressing clade B HIV-1  
Gag-Pol

<400> SEQUENCE: 31

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Phe Phe Arg Glu Asp Leu Ala Phe Leu Gln Gly Lys Ala Arg Glu Phe
 1           5           10           15
Ser Ser Glu Gln Thr Arg Ala Asn Ser Pro Thr Ile Ser Ser Glu Gln
 20           25           30
Thr Gly Ala Asn Ser Pro Thr Arg Arg Glu Leu Gln Val Trp Gly Arg
 35           40           45
Asp Asn Asn Ser Pro Ser Glu Ala Gly Ala Asp Arg Gln Gly Thr Val
 50           55           60
Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp Gln Arg Pro Leu Val Thr
 65           70           75           80
Ile Lys Ile Gly Gly Gln Leu Lys Glu Ala Leu Leu Asp Thr Gly Ala
 85           90           95
Asp Asp Thr Val Leu Glu Glu Met Ser Leu Pro Gly Arg Trp Lys Pro
 100          105          110
Lys Met Ile Gly Gly Ile Gly Gly Phe Ile Lys Val Arg Gln Tyr Asp
 115          120          125
Gln Ile Leu Ile Glu Ile Cys Gly His Lys Ala Ile Gly Thr Val Leu
 130          135          140
Val Gly Pro Thr Pro Val Asn Ile Ile Gly Arg Asn Leu Leu Thr Gln
 145          150          155          160
Ile Gly Cys Thr Leu Asn Phe Pro Ile Ser Pro Ile Glu Thr Val Pro
 165          170          175
Val Lys Leu Lys Pro Gly Met Asp Gly Pro Lys Val Lys Gln Trp Pro
 180          185          190
Leu Thr Glu Glu Lys Ile Lys Ala Leu Val Glu Ile Cys Thr Glu Met
 195          200          205
Glu Lys Glu Gly Lys Ile Ser Lys Ile Gly Pro Glu Asn Pro Tyr Asn
 210          215          220
Thr Pro Val Phe Ala Ile Lys Lys Lys Asp Ser Thr Lys Trp Arg Lys
 225          230          235          240
Leu Val Asp Phe Arg Glu Leu Asn Lys Arg Thr Gln Asp Phe Trp Glu
 245          250          255
Val Gln Leu Gly Ile Pro His Pro Ala Gly Leu Lys Lys Lys Lys Ser
 260          265          270
Val Thr Val Leu Asp Val Gly Asp Ala Tyr Phe Ser Val Pro Leu Asp
 275          280          285
Glu Asp Phe Arg Lys Tyr Thr Ala Phe Thr Ile Pro Ser Ile Asn Asn
 290          295          300
Glu Thr Pro Gly Ile Arg Tyr Gln Tyr Asn Val Leu Pro Gln Gly Trp
 305          310          315          320
Lys Gly Ser Pro Ala Ile Phe Gln Ser Ser Met Thr Lys Ile Leu Glu
 325          330          335
Pro Phe Lys Lys Gln Asn Pro Asp Ile Val Ile Tyr Gln Tyr Met Asn
 340          345          350
Asp Leu Tyr Val Gly Ser Asp Leu Glu Ile Gly Gln His Arg Thr Lys
 355          360          365
Ile Glu Glu Leu Arg Gln His Leu Leu Arg Trp Gly Leu Thr Thr Pro
 370          375          380
Asp Lys Lys His Gln Lys Glu Pro Pro Phe Leu Trp Met Gly Tyr Glu

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385	390	395	400
Leu His Pro Asp Lys Trp Thr Val Gln Pro Ile Val Leu Pro Glu Lys	405	410	415
Asp Ser Trp Thr Val Asn Asp Ile Gln Lys Leu Val Gly Lys Leu Asn	420	425	430
Thr Ala Ser Gln Ile Tyr Pro Gly Ile Lys Val Arg Gln Leu Cys Lys	435	440	445
Leu Leu Arg Gly Thr Lys Ala Leu Thr Glu Val Ile Pro Leu Thr Glu	450	455	460
Glu Ala Glu Leu Glu Leu Ala Glu Asn Arg Glu Ile Leu Lys Glu Pro	465	470	475
Val His Gly Val Tyr Tyr Asp Pro Ser Lys Asp Leu Ile Ala Glu Ile	485	490	495
Gln Lys Gln Gly Gln Gly Gln Trp Thr Tyr Gln Ile Tyr Gln Glu Pro	500	505	510
Phe Lys Asn Leu Lys Thr Gly Lys Tyr Ala Arg Met Arg Gly Ala His	515	520	525
Thr Asn Asp Val Lys Leu Leu Thr Glu Ala Val Gln Lys Ile Thr Thr	530	535	540
Glu Ser Ile Val Ile Trp Gly Lys Thr Pro Lys Phe Lys Leu Pro Ile	545	550	555
Gln Lys Glu Thr Trp Glu Thr Trp Trp Thr Glu Tyr Trp Gln Ala Thr	565	570	575
Trp Ile Pro Glu Trp Glu Phe Val Asn Thr Pro Pro Leu Val Lys Leu	580	585	590
Trp Tyr Gln Leu Glu Lys Glu Pro Ile Val Gly Ala Glu Thr Phe Tyr	595	600	605
Val Asp Gly Ala Ala Asn Arg Glu Thr Lys Leu Gly Lys Ala Gly Tyr	610	615	620
Val Thr Asn Lys Gly Arg Gln Lys Val Val Pro Leu Thr Asn Thr Thr	625	630	635
Asn Gln Lys Thr Gln Leu Gln Ala Ile Tyr Leu Ala Leu Gln Asp Ser	645	650	655
Gly Leu Glu Val Asn Ile Val Thr Asp Ser Gln Tyr Ala Leu Gly Ile	660	665	670
Ile Gln Ala Gln Pro Asp Lys Ser Glu Ser Glu Leu Val Asn Gln Ile	675	680	685
Ile Glu Gln Leu Ile Lys Lys Glu Lys Val Tyr Leu Ala Trp Val Pro	690	695	700
Ala His Lys Gly Ile Gly Gly Asn Glu Gln Val Asp Lys Leu Val Ser	705	710	715
Ala Gly Ile Arg Lys Ile Leu Phe Leu Asp Gly Ile Asp Lys Ala Gln	725	730	735

Asp Glu His

&lt;210&gt; SEQ ID NO 32

&lt;211&gt; LENGTH: 72

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
 pGAl and vaccine insert expressing clade B HIV-1  
 Gag-Pol

&lt;400&gt; SEQUENCE: 32

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Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser  
 1 5 10 15  
 Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe  
 20 25 30  
 His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly  
 35 40 45  
 Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr  
 50 55 60  
 His Gln Val Ser Leu Ser Lys Gln  
 65 70

<210> SEQ ID NO 33  
 <211> LENGTH: 25  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
 pGAl and vaccine insert expressing clade B HIV-1  
 Gag-Pol

<400> SEQUENCE: 33

Met Ala Gly Arg Ser Gly Asp Ser Asp Glu Asp Leu Leu Lys Thr Val  
 1 5 10 15  
 Arg Leu Ile Lys Phe Leu Tyr Gln Ser  
 20 25

<210> SEQ ID NO 34  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: synthetically generated peptide

<400> SEQUENCE: 34

Ser Ile Ile Asn Phe Glu Lys Leu  
 1 5

<210> SEQ ID NO 35  
 <211> LENGTH: 281  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
 pGAl and vaccine insert expressing clade B HIV-1  
 Gag-Pol

<400> SEQUENCE: 35

Met Arg Val Lys Glu Lys Tyr Gln His Leu Trp Arg Trp Gly Trp Arg  
 1 5 10 15  
 Trp Gly Thr Met Leu Leu Gly Met Leu Met Ile Cys Ser Ala Thr Glu  
 20 25 30  
 Lys Leu Trp Val Thr Val Tyr Tyr Gly Val Pro Val Trp Lys Glu Ala  
 35 40 45  
 Thr Thr Thr Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr Asp Thr Glu  
 50 55 60  
 Val His Asn Val Trp Ala Thr His Ala Cys Val Pro Thr Asp Pro Asn  
 65 70 75 80  
 Pro Gln Glu Val Val Leu Val Asn Val Thr Glu Asn Phe Asn Met Trp  
 85 90 95  
 Lys Asn Asp Met Val Glu Gln Met His Glu Asp Ile Ile Ser Leu Trp  
 100 105 110

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Asp Gln Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val Ser  
           115                                  120                                  125  
  
 Leu Lys Cys Thr Asp Leu Lys Asn Asp Thr Asn Thr Asn Ser Ser Ser  
           130                                  135                                  140  
  
 Gly Arg Met Ile Met Glu Lys Gly Glu Ile Lys Asn Cys Ser Phe Asn  
   145                                  150                                  155                                  160  
  
 Ile Ser Thr Ser Ile Arg Gly Lys Tyr Gln Lys Glu Tyr Ala Phe Phe  
                                   165                                  170                                  175  
  
 Tyr Lys Leu Asp Ile Ile Pro Ile Asp Asn Asp Thr Thr Ser Tyr Thr  
                                   180                                  185                                  190  
  
 Leu Thr Ser Cys Asn Thr Ser Val Ile Thr Gln Ala Cys Pro Lys Val  
           195                                  200                                  205  
  
 Ser Phe Glu Pro Ile Pro Ile His Tyr Cys Ala Pro Ala Gly Phe Ala  
           210                                  215                                  220  
  
 Ile Leu Lys Cys Asn Asn Lys Thr Phe Asn Gly Thr Gly Pro Cys Thr  
   225                                  230                                  235                                  240  
  
 Asn Val Ser Thr Val Gln Cys Thr His Gly Ile Arg Pro Val Val Ser  
                                   245                                  250                                  255  
  
 Thr Gln Leu Leu Leu Asn Gly Ser Leu Ala Glu Glu Glu Val Val Ile  
                                   260                                  265                                  270  
  
 Arg Ser Ser Asp Leu Glu Glu Glu Ile  
           275                                  280

<210> SEQ ID NO 36  
 <211> LENGTH: 21  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: tpa leader sequence of pGA1 and pGA2

<400> SEQUENCE: 36

Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu Leu Cys Gly  
   1                  5                                  10                                  15  
  
 Ala Val Phe Val Ser  
           20

<210> SEQ ID NO 37  
 <211> LENGTH: 3894  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: complementary strand of vaccine vector pGA1

<400> SEQUENCE: 37

acaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaagggcc gcgttgctgg 60  
 cgtttttcca taggtccgcg cccctgacg agcatcaca aaatcgacgc tcaagtcaga 120  
 ggtggcgaaa cccgacagga ctataaagat accaggcggt tccccctgga agctccctcg 180  
 tgcgctctcc tgttccgacc ctgccgctta cggatacct gtccgccttt ctcccttcgg 240  
 gaagcgtggc gctttctcat agctcacgct gtaggtatct cagttcgggt taggtcggtc 300  
 gctccaagct gggctgtgtg cacgaacccc cgttcagcc cgaccgctgc gccttatccg 360  
 gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg gcagcagcca 420  
 ctggtaacag gattagcaga gcgaggtatg taggcgggtg tacagagttc ttgaagtggg 480  
 ggcctaacta cggtacact agaagaacag tatttggtat ctgcgctctg ctgaagccag 540  
 ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc gctggtagcg 600

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gtgggttttt	tgtttgcaag	cagcagatta	cgcgagaaa	aaaaggatct	caagaagatc	660
ctttgatctt	ttctacgggg	tctgacgctc	agtggaaacga	aaactcacgt	taagggattt	720
tggtcatgag	attatcaaaa	aggatcttca	cctagatcct	tttcacgtag	aaagccagtc	780
cgagaaaacg	gtgctgaccc	cgatgaatg	tcagctactg	ggctatctgg	acaagggaaa	840
acgcaagcgc	aaagagaaa	caggtagctt	gcagtgggct	tacatggcga	tagctagact	900
gggcggtttt	atggacagca	agcgaaccgg	aattgccagc	tggggcgccc	tctggtaagg	960
ttgggaagcc	ctgcaaagta	aactggatgg	ctttcttgcc	gccaaagatc	tgatggcgca	1020
ggggatcaag	atctgatcaa	gagacaggat	gaggatcggt	tcgcatgatt	gaacaagatg	1080
gattgcacgc	aggttctccg	gccgcttggg	tggagaggct	attcggctat	gactgggcac	1140
aacagacaat	cggtctctct	gatgccgccc	tggtccggct	gtcagcgccg	gggcgcccgg	1200
ttctttttgt	caagaccgac	ctgtccgggt	ccctgaatga	actgcaagac	gaggcagcgc	1260
ggctatcgtg	gctggccacg	acgggcgttc	cttgccgagc	tgtgctcgac	gttgctcactg	1320
aagcgggaag	ggactggctg	ctattgggcy	aagtgccggg	gcaggatctc	ctgtcatctc	1380
accttgctcc	tgccgagaaa	gtatccatca	tggctgatgc	aatgcggcgg	ctgcatacgc	1440
ttgatccggc	tacctgccca	ttcgaccacc	aagcgaaaca	tcgcatcgag	cgagcacgta	1500
ctcggatgga	agccggtctt	gtcgatcagg	atgatctgga	cgaagagcat	caggggctcg	1560
cgccagccga	actgttcgcc	aggctcaagg	cgagcatgcc	cgacggcgag	gatctcgtcg	1620
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&lt;210&gt; SEQ ID NO 38

&lt;211&gt; LENGTH: 2947

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: complementary strand of vaccine vector pGA2

&lt;400&gt; SEQUENCE: 38

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&lt;210&gt; SEQ ID NO 39

&lt;211&gt; LENGTH: 3893

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: complementary strand of vaccine vector pGA3

&lt;400&gt; SEQUENCE: 39

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pGA2 and insert JS2 expressing clade HIV-1 VL

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<400> SEQUENCE: 40

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Val Glu Thr Glu Thr Glu Thr Asp Pro Cys Asp
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<210> SEQ ID NO 41
<211> LENGTH: 73
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protein encoded by construct of vaccine vector
pGA2 and insert JS2 expressing clade HIV-1 VL

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<400> SEQUENCE: 41

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 1           5           10           15

```

```

Leu Ser Thr Tyr Leu Gly Arg Ser Ala Glu Pro Val Pro Leu Gln Leu
 20           25           30

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Pro Pro Leu Glu Arg Leu Thr Leu Asp Cys Asn Glu Asp Cys Gly Thr
 35           40           45

```

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Ser Gly Ser Gln Gly Val Gly Ser Pro Gln Ile Leu Val Glu Ser Pro

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                                  Gag-Pol

<400> SEQUENCE: 42

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<210> SEQ ID NO 43  
 <211> LENGTH: 81  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: protein encoded by construct of vaccine vector  
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                                  Gag-Pol

<400> SEQUENCE: 43

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 20                      25                      30

Ala Glu Pro Val Pro Leu Gln Leu Pro Pro Leu Glu Arg Leu Thr Leu  
 35                      40                      45

Asp Cys Asn Glu Asp Cys Gly Thr Ser Gly Thr Gln Gly Val Gly Ser  
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Glu

<210> SEQ ID NO 44  
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 <213> ORGANISM: Artificial Sequence  
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 <223> OTHER INFORMATION: synthetically generated peptide

<400> SEQUENCE: 44

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<210> SEQ ID NO 45  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: HIV-1

<400> SEQUENCE: 45

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<210> SEQ ID NO 46  
 <211> LENGTH: 18  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:

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<223> OTHER INFORMATION: tpa leader sequence of pGA3

<400> SEQUENCE: 46

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1				5				10					15	

Val	Ser
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What is claimed is:

1. A vector comprising:
  - (a) a gene encoding kanamycin resistance;
  - (b) Col E1 replicator;
  - (c) a lambda T0 terminator operably linked to the gene encoding kanamycin resistance; and
  - (d) a eukaryotic transcription cassette comprising a CMV immediately early promoter, a polyadenylation signal sequence, and a nucleic acid sequence encoding: an HIV gag protein; an HIV pol protein fragment lacking the integrase domain and having amino acid changes at Asp185, Trp266, and Glu478; an HIV tat protein; an HIV rev protein; an HIV vpu protein; and an HIV env protein.
2. The vector of claim 1, wherein the HIV proteins are HIV clade B proteins.
3. The vector of claim 2, HIV Glade B proteins are HIV BH10 proteins or HIV ADA proteins.
4. The vector of claim 1, wherein the eukaryotic transcription cassette further comprises intron A of the CMV immediate early promoter.
5. The vector of claim 1, wherein the polyadenylation sequence is the rabbit beta globin polyadenylation sequence.
6. The vector of claim 1, wherein the polyadenylation sequence is the bovine growth hormone polyadenylation sequence.
7. A vector comprising the nucleotide sequence of SEQ ID NO:2.
8. The vector of claim 1, wherein Asp185 is changed to Asn, Trp266 is changed to Thr, and Glu478 is changed to Gln.
9. The vector of claim 1 or 8, wherein the HIV gag protein has amino acid changes at Cys392, Cys395, Cys413 and Cys416.
10. The vector of claim 9, wherein Cys392 is changed to Ser, Cys395 is changed to Ser, Cys413 is changed to Ser and Cys416 is changed to Ser.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,795,017 B2  
APPLICATION NO. : 11/009063  
DATED : September 14, 2010  
INVENTOR(S) : Harriet L. Robinson et al.

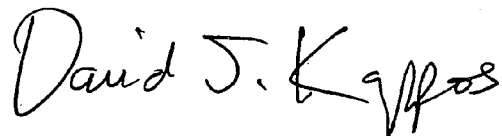
Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page, Column 2 (Abstract), line 12-13, delete “histocompatability” and insert  
-- histocompatibility --

Signed and Sealed this

Fourteenth Day of December, 2010

A handwritten signature in black ink that reads "David J. Kappos". The signature is written in a cursive, flowing style with a large, stylized 'D' and 'K'.

David J. Kappos  
*Director of the United States Patent and Trademark Office*